BIOLOGICALLY-ACTIVE DNA-BINDING SITES AND RELATED METHODS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of the filing date of U.S. Application No.

5   60/604,470, filed August 25, 2004, entitled "BIOLOGICALLY-ACTIVE DNA-
BINDING SITES AND RELATED METHODS", and of U.S. Application
No.60/550,074 filed March 4, 2004, entitled "TRANSCRIPTIONAL
REGULATORY CODES OF EUKARYOTIC GENOMES AND METHODS
THEREOF." The entire teachings of the referenced applications are incorporated by

10 reference herein.

STATEMENT REGARDING FEDERALLY-SPONSORED RESEARCH OR DEVELOPMENT

The invention described herein was supported, in whole or in part, by the

15 National Institute of Health grant no. HG002668. The United States government has
certain rights in the invention.

BACKGROUND OF THE INVENTION

Genome sequences contain information necessary to control gene expression

20 programs and specify protein and other gene products. DNA-binding transcriptional
regulators interpret the genome's regulatory code by binding to specific sequences to
induce or repress gene expression (Jacob *et al.* *J Mol Biol* 3, 318-56 (1961), Kellis
et al. *Nature* 423, 241-54 (2003), Cliften *et al.* *Science* 301, 71-6 (2003)).
Substantial portions of genome sequence are believed to be regulatory (Pritsker *et al.*

25 *Genome Res* 14, 99-108 (2004); Wang, *et al.* *Bioinformatics* 19, 2369-80 (2003);
Blanchette *et al.* *Nucleic Acids Res* 31, 3840-2 (2003); Iyer *et al.* *Nature* 409, 533-
8. (2001); Ren *et al.* *Science* 290, 2306-9. (2000)), but the DNA sequences that
actually contribute to the regulatory code are ill-defined. In contrast, the triplet code
used to translate nucleotide sequences into protein molecules is well known (Lee *et*

30 *al.* *Science* 298, 799-804. (2002), Lieb *et al.* *Nat Genet* 28, 327-34 (2001), Roth *et*
al. *Nat Biotechnol* 16, 939-45. (1998)). Knowledge of the genome's transcriptional
regulatory code could provide new insights into the principles that govern global

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gene regulation.

Comparative genomics has recently been used to identify functional sequence elements in the yeast genome (Pritsker *et al. Genome Res* 14, 99-108 (2004), Wang, *et al. Bioinformatics* 19, 2369-80 (2003), Liu *et al. Nat Biotechnol* 20, 835-9 (2002), Bailey *et al. Proc Int Conf Intell Syst Mol Biol* 3, 21-9 (1995)). Comparative analysis of the genome sequences of multiple yeast species revealed phylogenetically-conserved sequences, and these sequences were used to facilitate identification of genes and putative regulatory elements. Conserved sequence information alone does not reveal, however, the subset of sequences that are bound by transcriptional regulators, the identity of the binding regulators, or the conditions under which the regulators occupy their binding sites.

Therefore, there is a need to develop novel methods and algorithms for identifying the biologically- active DNA-binding site bound by transcriptional regulators *in vivo*.

BRIEF SUMMARY OF THE INVENTION

The invention provides novel methods relating to biologically-active DNA-binding sites for a protein of interest. One aspect of the invention provides methods for identifying the biologically-active DNA-binding sites for a protein of interest in the genome of a cell, such as a living cell. In embodiments of the invention, the protein of interest is a transcriptional regulator, a protein for mediating DNA recombination, a protein for mediating DNA repair, a protein for mediating DNA modification, or a protein for mediating DNA replication. One aspect of the invention also provides methods for identifying the DNA-sequence motif for a protein of interest.

The invention also provides methods of identifying agents, such as antisense agents, antibodies, polypeptides or small molecules, which alter the set of biologically-active DNA-binding sites to which a protein of interest binds in a cell. Such agents may be used therapeutically, particularly where binding, or lack of

binding, of a protein, such as a transcriptional regulator, to the genome of a cell results in disease. The invention further provides methods of identifying conditions or cellular genotypes where the protein of interest differentially binds to the genome of a cell.

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The invention further provides methods for identifying cellular pathways that are regulated by the protein of interest, including biochemical, regulatory, gene expression and signaling pathways. The invention further provides methods to identify a property of a protein of interest, such as a transcriptional regulator, which

10   correlates with differential binding of the protein of interest to the genome of a cell.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1B show a general strategy for discovering binding site specificities for yeast transcriptional regulators. Figure 1 A: Cis-regulatory sequences

15   that likely serve as recognition sites for transcriptional regulators were identified by combining information from genome-wide location data, phylogenetically conserved sequences, and previously published data, as described in the Examples. The compendium of regulatory sequence motifs can be found in Table 3. Figure 1B: Selected sequence specificities that were "rediscovered" and were newly discovered

20   are displayed. The total height of the column is proportional to the information content of the position, and the individual letters have height proportional to the product of their frequency and the information content (Schneider et al. Nucleic Acids Res 18, 6097-100 (1990).

25   Figures 2A-2C show the drafting of the yeast transcriptional regulatory map. Figure 2A: Portions of chromosomes illustrating locations of genes (large rectangles) and conserved DNA sequences (small boxes) bound in vivo by transcriptional regulators. Figure 2B: Combining binding data and sequence conservation data. The diagram depicts all sequences matching a motif from the

30   compendium (top), all such conserved sequences (middle) and all such conserved sequences bound by a regulator (bottom). Figure 2C: Regulator binding site distribution. The line labeled "actual" shows the distribution of distances from the

start codon of open reading frames to binding sites in the adjacent upstream region. The line labeled "randomized" represents a randomized distribution.

Figure 3 shows yeast promoter architectures. Single regulator architecture:
5   promoter regions that contain one or more copies of the binding site sequence for a single regulator. Repetitive motif architecture: promoter regions that contain multiple copies of a binding site sequence of a regulator. Multiple regulator architecture: promoter regions that contain one or more copies of the binding site sequences for more than one regulator. Co-occurring regulator architecture:
10   promoters that contain binding site sequences for recurrent pairs of regulators. For the purposes of illustration, not all sites are shown and scale is approximate. Additional information can be found in Tables 4-6.

Figure 4 shows environment-specific utilization of the transcriptional
15   regulatory code. Four patterns of genome-wide binding behavior are depicted in a graphic representation on the left, where transcriptional regulators are represented by circles and are placed above and below a set of target genes/promoters. The lines between the regulators and the target genes/promoters represent binding events. Specific examples of the environment-dependent behaviors are depicted on the right.
20   Circles represent regulators and boxes represent their DNA binding sequences within specific promoter regions. Applicants note that regulators may exhibit different behaviors when different pairs of conditions are compared.

Figure 5 shows the distribution of the number of promoter regions bound per
25   regulator as a line labelled "actual". For regulators profiled under multiple conditions, the union of promoter regions bound under all conditions is reported. An average of randomized distributions for the same set of P values randomly assigned among regulators and promoter regions is shown as a line labeled "randomized".

30   Figure 6 shows an overview of motif discovery and assignment. Motifs were identified by applying a suite of motif discovery programs to the intergenic sequences identified by the binding data. The resulting specificity predictions were

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filtered for significance and then clustered to yield representative motifs. Conservation-based metrics were used to identify the highest-confidence subset of these motifs. For cases in which multiple significant binding motifs were found for a factor, Applicants used statistical scores or information from specificity databases to

5   choose a single motif for each regulator. A complete description of the method can be found in experimental procedures.

Figure 7 shows a comparison of Cin5 binding to two sequences. Recombinant Cin5 was purified from bacteria and incubated with a Cy5-labeled

10   oligonucleotide containing the sequence (gcgacaTTACCTAAgggc) and challenged with one of two unlabeled competitors: the same sequence (lanes 2-8) or the previously published binding site (gcgacaTTACTAAagggc; lanes 9-15). The concentration of each competitor was varied in 3-fold steps. The probe based on our discovered motif was approximately 27-fold better in competing away the shifted

15   band compared to the probe based on the previously published specificity. Similar results were obtained for a probe containing a core sequence of TTACGTAA.

Figure 8 shows a pairwise comparison of the number of promoter regions bound under two different conditions for 25 regulators (based solely on genome-

20   wide location data with P< 0.001). Dark bars represent the number of promoter regions bound under growth in rich medium; light bars represent the number of promoter regions bound under growth in amino acid starvation medium.

Figure 9 shows the quality of Gcn4 binding sites among intergenic regions

25   bound under different conditions. Each intergenic region was scored based on the quality of the best matching subsequence to the Gcn4 binding specificity (TGASTCA). In rich media conditions 68% of the intergenic regions contain high-quality matches to the Gcn4 specificity. Under starvation conditions the levels of Gcn4 protein rise, and the set of bound intergenic regions expands. Of the newly

30   bound regions, only 27% contain high-quality matches. By contrast, only 3% of all intergenic regions contain matches of this quality.

DETAILED DESCRIPTION OF THE INVENTION

I. Overview

The invention provides, in part, novel methods relating to the biologically-active DNA-binding sites. One aspect of the invention provides a method of

5   identifying a set of biologically-active DNA-binding sites for a protein of interest in the genome of a cell, the method comprising (i) identifying a set of regions of genomic DNA to which the protein of interest is bound in the cell; (ii) identifying candidate DNA-binding sites in the identified regions of genomic DNA, wherein a candidate DNA-binding site comprises a sequence corresponding to a DNA-

10   sequence motif for the protein of interest; (iii) determining if the candidate DNA-binding sites are conserved in an equivalent genomic region in one or more different species from that of the cell, wherein a candidate DNA-binding site that is conserved in at least one species is said to be a biologically-active DNA-binding site. In one embodiment, step (i) further comprises identifying a DNA-sequence motif for the

15   protein of interest from the set of regions of genomic DNA. In a specific embodiment, the DNA-sequence motif is enriched by a statistically-significant amount in the set of regions of genomic DNA relative to a suitable control. The term "statistically significant" as used herein means that the obtained results are not likely to be due to chance fluctuations at the specified level of probability. The two

20   most commonly specified levels of significance are 0.05 (p=0.05) and 0.01 (p=0.01). The level of significance equal to 0.05 and 0.01 means that the probability of error is 5 out of 100 and 1 out of 100, respectively. However, higher levels of significance may be used in connection with the methods described herein, such as 0.005, 0.001, 0.0001, 0.00001, or intermediate values.

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In one embodiment, the suitable control comprises a set of genomic regions which are not bound by the protein of interest in the cell. In another embodiment, the suitable control comprises a set of randomly selected genomic regions. In another embodiment, the suitable control comprises a set of randomly-generated sequences.

30   In another embodiment, the suitable control comprises a set of genomic regions which are bound by a mutant form of the protein of interest in the cell.

In one embodiment, the candidate DNA-binding site is conserved if the equivalent genomic region in at least one different species comprises a nucleic acid sequence that matches the DNA-sequence motif for the protein of interest. In another embodiment, the DNA-sequence motif is identified using at least one

5   algorithm. In another embodiment, the DNA-sequence motif is identified using a combination of algorithms. In one embodiment, the algorithm is selected from the group consisting of AlignACE, MEME, MDscan, the Kellis Method, Mogul, Verbumculus, YMF, BioProspector, Motif Sampler and SUPERPOSITION.

10   In one embodiment of the methods described herein for identifying a set of biologically-active DNA-binding sites, the regions of genomic DNA comprise promoter regions. In another embodiment, the regions of genomic DNA have a length from about between 50 bp to about 10 kb. In another embodiment, step (i) comprises performing genome-wide location analysis (GWLA) of the protein of

15   interest. In a specific embodiment, the GWLA comprises ChIP-chip, however, any method for identifying binding sites for proteins of interest may be used . In another embodiment, the candidate DNA-binding site is less that 20 bp in length.

In one embodiment, the DNA-sequence motif is degenerate in at least one

20   position. In another embodiment, one or more of the different species is classified in the same genus as the cell. In another embodiment, step (iii) comprises determining if the candidate DNA-binding sites are conserved in equivalent genomic regions in two or more different species. In another embodiment, the protein of interest is a transcriptional regulator. In a specific embodiment, the protein of interest comprises

25   a DNA binding domain. In another embodiment, the protein of interest does not comprise a DNA-binding domain. In a specific embodiment, the DNA-binding domain is selected from the group consisting of zinc finger, winged-helix, leucine zipper, homeodomain and helix-loop-helix (HLH). In another embodiment, the protein of interest is a protein for mediating DNA recombination, a protein for

30   mediating DNA repair, a protein for mediating DNA modification, or a protein for mediating DNA replication.

In one embodiment, the set of biologically-active DNA—binding sites
comprises one or more biologically-active DNA-binding sites. In another
embodiment, the set of biologically-active DNA-binding sites comprises 10 or more
biologically-active binding sites. In another embodiment, two regions of genomic

5   DNA are equivalent if they both comprise a sequence of at least one orthologous
gene. In another embodiment, two regions of genomic DNA, each comprising an
intergenic region which is flanked by a first and a second open reading frame (ORF)
in their respective genomes, are said to be equivalent if (i) the first ORF in the two
regions are orthologous ORFs and (ii) if the second ORFs in the two regions are

10 orthologous ORFs.

In one embodiment, the cell is a eukaryotic cell, such as a mammalian cell,
and more preferably, a human cell. In another embodiment, the cell is a primary
cell, such as from a tissue biopsy. In a specific embodiment, the tissue biopsy is

15 isolated from a subject afflicted with a disorder. In yet another embodiment, the cell
is a single-cell organism, such as a yeast cell. In one embodiment, the cell is a stem
cell. The term "stem cell" as used herein refers to a cell that gives rise to a lineage
of cells, and that may be characterized as a cell that upon division, produces
dissimilar daughters, one replacing the origin or partially differentiated stem cells.

20 Stem cells include embryonic stem cells, umbilical cord blood stem cells, and
adult/peripheral stem cells.

Another aspect of the invention provides a method of identifying an agent
which alters the set of biologically-active DNA-binding sites for a protein of interest

25 in the genome of a cell, the method comprising (i) contacting an experimental cell
with a candidate agent; (ii) identifying a set of biologically-active DNA-binding
sites for a protein of interest in the genome of the experimental cell of step using any
of the methods described herein, thereby generating an experimental set of
biologically-active DNA-binding sites; (iii) comparing (1) the experimental set of

30 biologically-active DNA-binding sites to (2) a control set of biologically-active
DNA-binding sites for the protein of interest; wherein a candidate agent is identified
if the experimental set and the control set differ. In one embodiment, the control set

is derived from a control cell that is not contacted with the candidate agent.

Yet another aspect of the invention provides a method of identifying a
pathway that is transcriptionally regulated by a protein of interest in a cell, the
5   method comprising (i) identifying a set of biologically-active DNA-binding sites for
a protein of interest in the genome of the cell according to any of the methods
described herein; and (ii) identifying at least two candidate genes likely to be
regulated by binding of the protein of interest to the set of biologically-active DNA-
binding sites identified in (i); wherein the pathway that is transcriptionally regulated
10   by a protein of interest is identified if at least two candidate genes are members of
the same pathway. In a specific embodiment, a pathway that is transcriptionally
regulated by a protein of interest is identified if at least 2, 3, 4 or 5 candidate genes
are members of the same pathway. In one embodiment, the pathway is a
biochemical pathway. In another embodiment, the pathway is a gene expression
15   pathway. In another embodiment, the pathway is a regulatory pathway. In another
embodiment, the candidate gene is likely regulated by the protein of interest if the
promoter for the candidate gene comprises at least one biologically-active DNA-
binding site. In one embodiment, the promoter region of a candidate gene comprises
from 3kb 5' to 1kb 3' of the transcription initiation site. In still another embodiment,
20   the invention provides a method of modulating a pathway that is transcriptionally
regulated by a protein of interest by exposing a cell to an agent or condition which
alters the binding sites to which the protein of interest binds, as discussed further
below.

25   Another aspect of the invention provides a method of identifying two sets of
conditions in which a protein of interest differentially binds to the genome of a cell,
the method comprising: (i) identifying a first set of biologically-active DNA-binding
sites for the protein of interest in the genome of a cell according to any one of the
methods described herein, wherein the cell is exposed to a first set of conditions; (ii)
30   identifying a second set of biologically-active DNA-binding sites for the protein of
interest in the genome of a cell according to any one of the methods described herein
for identifying a set of biologically-active DNA-binding sites, wherein the cell is

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exposed to a second set of conditions; and (iii) comparing the first set of biologically-active DNA-binding sites to the second set of biologically-active DNA-binding sites and determining if the two sets differ.

5   Another aspect of the invention provides a method of identifying a property of a gene product of a gene of interest that correlates with the binding activity of a polypeptide encoded by the gene of interest to the genome of a cell, the method comprising (i) identifying two sets of conditions in which a protein of interest differentially binds to the genome of the cell; (ii) determining a property of a gene

10   product of the gene of interest in (a) a cell exposed to a first set of conditions; and in (b) a cell exposed to a second set of conditions; and (iii) determining if at least one property of the gene product differs in the two cells of step (ii), thereby identifying a property that correlates with the binding activity of a gene of interest to the genome of a cell.

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          A related aspect of the invention provides a method of identifying a property of a gene product of a gene of interest that correlates with the binding activity of a polypeptide encoded by the gene of interest to the genome of a cell, the method comprising (i) identifying an agent which alters the set of biologically-active DNA-

20   binding sites for a protein of interest in the genome of a cell; (ii) determining a property of a gene product of the gene of interest in (a) a cell contacted with the agent; and in (b) a cell not contacted with the agent; and (iii) determining if at least one property of the gene product differs in the two cells of step (ii), thereby identifying a property that correlates with the binding activity of a gene of interest to

25   the genome of a cell.

          In one embodiment, the property is selected from the group consisting of a protein modification, expression level, enzymatic activity and intracellular localization. In one embodiment, the expression product is an mRNA or a

30   polypeptide. In another embodiment, the property comprises the expression level of the gene product.

In another embodiment, the property comprises the subcellular localization of the gene product. In another embodiment, the property comprises the phosphorylation state of gene product. In another embodiment, the property comprises the molecular weight of the gene product. In another embodiment, the

5   property comprises the isoelectric point of the gene product. In another embodiment, the property comprises the nucleic acid sequence or the amino acid sequence of the gene product. In another embodiment, the property comprises the physical association of the protein of interest with another polypeptide.

10   Another aspect of the invention provides a method of identifying two cell genotypes in which a protein of interest differentially binds to the genome of a cell, the method comprising: (i) identifying a first set of biologically-active DNA-binding sites for the protein of interest in the genome of a cell of a first genotype; (ii) identifying a second set of biologically-active DNA-binding sites for the protein of

15   interest in the genome of a cell of a second genotype; (iii) comparing the first set of biologically-active DNA-binding sites to the second set of biologically-active DNA-binding sites and determining if the two sets differ.

20   II. Definitions

For convenience, certain terms employed in the specification, examples, and appended claims, are collected here. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

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The articles "a" and "an" are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

30   The term "including" is used herein to mean, and is used interchangeably with, the phrase "including but not limited" to.

The term "or" is used herein to mean, and is used interchangeably with, the term "and/or," unless context clearly indicates otherwise.

The term "such as" is used herein to mean, and is used interchangeably, with

5 the phrase "such as but not limited to".

A "patient" or "subject" to be treated by the method of the invention can mean either a human or non-human animal, preferably a mammal.

10 The term "encoding" comprises an RNA product resulting from transcription of a DNA molecule, a protein resulting from the translation of an RNA molecule, or a protein resulting from the transcription of a DNA molecule and the subsequent translation of the RNA product.

15 The term "promoter" is used herein to mean a DNA sequence that initiates the transcription of a gene. Promoters are typically found 5' to the gene and located proximal to the start codon. If a promoter is of the inducible type, then the rate of transcription increases in response to an inducer. Promoters may be operably linked to DNA binding elements that serve as binding sites for transcriptional regulators.

20 The term "mammalian promoter" is used herein to mean promoters that are active in mammalian cells. Similarly, "prokaryotic promoter" refers to promoters active in prokaryotic cells.

The term "expression" is used herein to mean the process by which a

25 polypeptide is produced from DNA. The process involves the transcription of the gene into mRNA and the translation of this mRNA into a polypeptide. Depending on the context in which used, "expression" may refer to the production of RNA, protein or both.

30 The term "recombinant" is used herein to mean any nucleic acid comprising sequences which are not adjacent in nature. A recombinant nucleic acid may be generated *in vitro*, for example by using the methods of molecular biology, or *in*

vivo, for example by insertion of a nucleic acid at a novel chromosomal location by homologous or non-homologous recombination.

5   The term "transcriptional regulator" refers to a biochemical element that acts to prevent or inhibit the transcription of a promoter-driven DNA sequence under certain environmental conditions (*e.g.*, a repressor or nuclear inhibitory protein), or to permit or stimulate the transcription of the promoter-driven DNA sequence under certain environmental conditions (*e.g.*, an inducer or an enhancer).

10   The term "microarray" refers to an array of distinct polynucleotides or oligonucleotides synthesized on a substrate, such as paper, nylon or other type of membrane, filter, chip, glass slide, or any other suitable solid support.

       The terms "disorders" and "diseases" are used inclusively and refer to any
15   deviation from the normal structure or function of any part, organ or system of the body (or any combination thereof). A specific disease is manifested by characteristic symptoms and signs, including biological, chemical and physical changes, and is often associated with a variety of other factors including, but not limited to, demographic, environmental, employment, genetic and medically
20   historical factors. Certain characteristic signs, symptoms, and related factors can be quantitated through a variety of methods to yield important diagnostic information.

       The term "modulation" refers to upregulation (*i.e.*, activation or stimulation), downregulation (*i.e.*, inhibition or suppression) of a response, or the two in
25   combination or apart. A "modulator" is a compound or molecule that modulates, and may be, *e.g.*, an agonist, antagonist, activator, stimulator, suppressor, or inhibitor.

       The term "agonist" refers to an agent that mimics or up-regulates (*e.g.*, potentiates or supplements) the bioactivity of a protein, *e.g.*, polypeptide X. An
30   agonist may be a wild-type protein or derivative thereof having at least one bioactivity of the wild-type protein. An agonist may also be a compound that upregulates expression of a gene or which increases at least one bioactivity of a

protein. An agonist may also be a compound which increases the interaction of a polypeptide with another molecule, *e.g.*, a target peptide or nucleic acid.

5   The term "antagonist" refers to an agent that downregulates (*e.g.*, suppresses or inhibits) at least one bioactivity of a protein. An antagonist may be a compound which inhibits or decreases the interaction between a protein and another molecule, *e.g.*, a target peptide or enzyme substrate. An antagonist may also be a compound that downregulates expression of a gene or which reduces the amount of expressed protein present.

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The term "intergenic region" as used herein refer to genomic DNA sequence which lie between adjacent open-reading frames (ORFs). Intergenic regions may comprise regulatory elements such as promoter, operators or enhancers, although regulatory sequences can also be located in coding regions.

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III. Methods of Identifying Biologically-Active DNA-Binding Sites
One aspect of the invention provides methods of identifying a set of biologically-active DNA-binding sites for a protein of interest in the genome of a cell. One specific aspect method comprises (i) identifying a set of regions of

20   genomic DNA to which the protein of interest is bound in the cell; (ii) identifying candidate DNA-binding sites in the identified regions of genomic DNA, wherein a candidate DNA-binding site comprises a sequence corresponding to a DNA-sequence motif for the protein of interest;(iii) determining if the candidate DNA-binding sites are conserved in an equivalent genomic region in one or more different

25   species from that of the cell, wherein a candidate DNA-binding site that is conserved in at least one species is a biologically-active DNA-binding site.

The methods of the present invention are not limited to any particular type of cell. The cell may be, for example, a prokaryotic cell or an eukaryotic cell. In one

30   preferred embodiment, the cell is a mammalian cell. Other preferred cells include primate, and rodent cells, such as human and mouse cells, and cells from model organisms such as yeast, zebrafish, *C. elegans* or *Drosophila*. The cells used in

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methods of the invention may be cells that have been passaged extensively *in vitro*, including immortalized cell lines such as cancer cell lines. Cell lines that may be used in the present invention also include stem cells, such as embryonic and adult stem cells.

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In other embodiments, the cells are primary cells which have undergone minimal, if any, culturing *in vitro*. In one exemplary embodiment, the cells are derived from freshly isolated tissue, such as from a tissue biopsy. Such cells are preferred in some embodiments as the patterns of gene expression in such cells

10   would be expected to most closely resemble the *in vivo* state. In some embodiments, the cells are derived from a subject afflicted with a disorder. Such cells may provide insights into the disease state and aid in the identification of therapeutics to treat or prevent the disorder.

15   In some embodiments, the cells are of a single cell type, while other embodiments may include cells of more than one cell type. For instance, one embodiment may use only hepatocytes, whereas other embodiments may use hepatocytes, neurons and pancreatic beta cells. In other embodiments, the cells may include cells from an entire organism or combination of organisms.

20

The protein of interest in the methods described herein may associate directly or indirectly with DNA. In some embodiments, the protein of interest comprises a DNA-binding domain which may allow direct binding to DNA molecules. Exemplary DNA-binding domains include zinc finger, winged-helix, leucine zipper,

25   homeodomain or helix-loop-helix (HLH). In other embodiments, the protein of interest lacks a DNA-binding domain. In certain embodiments of the invention, the protein of interest comprises specific transcription factors, coactivators, corepressors or complexes thereof. Transcription factors bind to specific cognate DNA elements such as promoters, enhancers and silencer elements, and are responsible for

30   regulating gene expression. Transcription factors may be activators of transcription, repressors of transcription or both, depending on the cellular context. In one embodiment, the protein of interest is any one of the transcriptional regulators listed

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in the TRANSFAC database (See E. Wingender, X. Chen, R. Hehl, H. Karas, I. Liebich, V. Matys, T. Meinhardt, M. Pr, I. Reuter, and F. Schacherer. TRANSFAC: an integrated system for gene expression regulation. Nucleic Acids Res., 28:316–319, 2000).

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Transcription factors may belong to any class or type of known or identified transcription factor. Examples of known families or structurally-related transcription factors include helix-loop-helix, leucine zipper, zinc finger, ring finger, and hormone receptors. Transcription factors may also be selected based upon their

10   known association with a disease or the regulation of one or more genes. For example, transcription factors such as c-myc, Rel/Nf-kB, neuroD, c-fos, c-jun, and E2F may be targeted. Antibodies directed to any transcriptional coactivator or corepressor may also be used according to the invention. Examples of specific coactivators include CBP, CTIIA, and SRA, while specific examples of corepressors

15   include the mSin3 proteins, MITR, and LEUNIG. Furthermore, the genes regulated by proteins associated with transcriptional complexes, such as the histone acetylases (HATs) and histone deacetylases (HDACs), may also de determined using the methods described herein.

20   In other embodiments of the methods described herein, the protein of interest is a basal transcription factor or a component of the basal transcription machinery. In specific embodiments, components of the basal transcription machinery comprise RNA polymerases, including polI, polII and polIII, TBP, NTF-1 and Sp1 and any other component of TFIID, including, for example, the TAFs (e.g. TAF250,

25   TAF150, TAF135, TAF95, TAF80, TAF55, TAF31, TAF28, and TAF20), or any other component of a polymerase holoenzyme.

In some embodiments of the methods described herein, the protein of interest is native to the cell. By native it is meant that the protein of interest naturally occurs

30   in the cell. In other embodiments, the protein of interest is not native to the cell and is instead introduced through manipulation of the cell, including microinjection of the protein, liposome-mediated delivery of the protein (Weiner et al., (1994)

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Immunomethods; 4(3):201-9), or fusion to a polypeptide which allows entry into the cell, such as fusion to a Tat polypeptide (Becker-Hapak *et al.*, (2001) Methods. 24(3):247-56). In more preferred embodiments, the non-native protein is introduced into the cell through standard genetic engineering and recombinant DNA techniques.

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In some embodiments, the protein of interest is native to a cell of a different species from that of the cell used in the instant methods. In some embodiments, the protein of interest is a viral protein. In such embodiments, a cell may be contacted with a virus under conditions which allow infection of the cell by the virus in order

10　　to allow expression of the viral protein in the cell. In some embodiments, the gene encoding the protein of interest carries missense mutations, nonsense mutations, deletions, insertions or inversions such that a mutant protein may be expressed in the cell. In some embodiments, the protein of interest is a fusion protein. In one specific embodiment, the fusion protein comprises a transcriptional regulator fused

15　　to a "tag" which allows visualization or purification of the protein. Preferred tags include GFP polypeptides, GST domains, myc tags, His tags, or any other tag which allows the immunoprecipitation of the protein of interest when complexed with chromatin. In other embodiments, the protein-of-interest comprise artificial transcriptional regulators (see WO02/31166).

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In some embodiments of the methods described herein, the protein of interest has been causally implicated in a disease or disorder. Examples of diseases and transcriptional regulators which may cause them may be found in the scientific and medical literature, including in Medical Genetics, L.V. Jorde *et al.*, Elsevier Science

25　　2003, and Principles of Internal Medicine, 15th edition, ed by Braunwald *et al.*, McGraw-Hill, 2001; American Medical Association Complete Medical Encyclopedia (Random House, Incorporated, 2003); and The Mosby Medical Encyclopedia, ed by Glanze (Plume, 1991). In some embodiments, the disorder is characterized by impaired function of at least one of the following organs or tissues:

30　　brain, spinal cord, heart, arteries, esophagus, stomach, small intestine, large intestine, liver, pancreas, lungs, kidney, urinary tract, ovaries, breasts, uterus, testis, penis, colon, prostate, bone, scalp, muscle, cartilage, thyroid gland, adrenal gland,

pituitary, bone marrow, blood, thymus, spleen, lymph nodes, skin, eye, ear, nose, teeth or tongue.

5   Biologically-active DNA-binding sites include sites on the genomic DNA of the cell to which the protein of interest binds. One skilled in the art would appreciate that while many sequences in a genome may match a DNA-sequence motif for the protein of interest, many of these sites are not bound *in vivo* by the protein of interest, and thus would not be considered biologically-active DNA-binding sites.

10

       In one embodiment, a biologically-active DNA-binding site comprises the minimal DNA sequence required for specific binding of the protein of interest to the DNA. In another embodiment, the biologically-active DNA-binding site comprises the stretch of DNA sequence that is physically contacted by the protein of interest

15   when the protein of interest is bound to DNA under native or physiological conditions such as in a live cell. In another embodiment, the biologically-active DNA-binding site comprises the promoter region, or any other functionally defined regulatory region for a gene, to which a protein of interest is bound to under native conditions in a cell.

20

       In some embodiments of the methods described herein, the set of biologically-active DNA-binding sites comprises one biologically-active DNA-binding site. In another embodiment, the set includes at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 20, 25, 30, 40 or 50 biologically-active DNA-binding sites. In some

25   embodiments, the set comprises the biologically-active DNA-binding site bound by a protein of interest when the cell is grown under one set of conditions, while in other embodiments the set may comprise more than one set of conditions. Accordingly, the set may be a "snapshot" of the sites bound by the protein of interest, or it may be a cumulative set of various snapshots, each taken under a

30   different set of conditions. The set may comprise the sites bound by the protein of interest in one cell type or in more than one cell type. For example, the set may comprises the sites bound by E2F in hepatocytes, or it may comprise the sites bound

in hepatocytes, adipocytes, neurons. In some embodiments, the set may comprise
the binding sites for a protein of interest in an organism. For example, the set may
comprise the sites for a transcription factor in *C. elegans*, such as the set that may be
obtained when whole worms are used as starting materials. In another embodiment,

5   the set comprises the binding sites located in promoter regions of at least 5%, 10%,
20%, 30%, 50%, 70%, 80%, 90%, 95%, 98% or 99% of the genes in the genome of
the cell.

        The identification of a set of regions of genomic DNA to which the protein

10   of interest is bound in the cell may be achieved using any technique know in the art.
In a preferred embodiments, the genomic regions are identified using genome-wide
location analysis (GWLA). GWLA has been described in International PCT
application Nos. WO01/16378 and WO02/059371, and U.S. Patent No.: 6,410,243.
In a preferred embodiment, the identification of the set of regions of genomic DNA

15   to which the protein of interest is bound in the cell comprises chromatin-
immunoprecipitation (ChIP) and subsequent analysis on DNA microarray (ChIP-
chip analysis) (see Buck and Lieb, (2004) Genomics 83:349-360; Ren *et al.* (2004)
Methods Enzymol. 376:304-315; Urnov *et al.* (2003) *Journal of Cellular
Biochemistry* 88:684-694; and Orlando, Valerio (2000) *TIBS* 25:99-103).

20

        In a preferred embodiment, the regions of genomic DNA, *i.e.* chromatin
fragments, bound by the protein of interest are isolated using chromatin
immunoprecipitation (ChIP). Briefly, this technique involves the use of a specific
antibody to immunoprecipitate chromatin complexes comprising the corresponding

25   antigen *i.e.* the transcriptional regulator, and examination of the nucleotide
sequences present in the immunoprecipitate. Immunoprecipitation of a particular
sequence by the antibody is indicative of interaction of the antigen with that
sequence. See, for example, O'Neill *et al.* in *Methods in Enzymology,* Vol. 274,
Academic Press, San Diego, 1999, pp. 189-197; Kuo *et al.* (1999) *Method* 19:425-

30   433; and Ausubel *et al.*, supra, Chapter 21. Accordingly, in one embodiment, the
DNA fragment bound the protein of interest is identified using an antibody which
binds to the protein of interest.

In one embodiment of the methods described herein, the protein of interest is crosslinked to chromatin (Solomon, M.J. and Varshavsky, A., Proc. Natl. Sci. USA 82:6470-6474; Orlando, V., TIBS, 25:99-104). There are a variety of methods

5   which can be used to link a DNA binding protein of the cell to the genome of the cell. For example, UV light can be used. In a particular embodiment, formaldehyde is used to crosslink DNA binding proteins to the genomic DNA of a cell. The chromatin may then be sheared, such as by sonication, to generate fragments of chromatin bound to the protein of interest.

10

In one embodiment, the chromatin immunoprecipitation technique is applied as follows. Cells which express the protein of interest, such as a native transcriptional regulator or a recombinant transcriptional regulator, are treated with an agent that crosslinks the transcriptional regulator to chromatin if that

15   transcriptional regulator is stably bound to it. The transcriptional regulator can be crosslinked to chromatin by, for example, formaldehyde treatment or ultraviolet irradiation. Subsequent to crosslinking, cellular nucleic acid is isolated, fragmented and incubated in the presence of an antibody directed against the transcriptional regulator. Antibody-antigen complexes are precipitated, crosslinks are reversed (for

20   example, formaldehyde-induced DNA-protein crosslinks can be reversed by heating) so that the sequence content of the immunoprecipitated DNA is tested for the presence of a specific sequence, for example, promoter regions. The antibody may bind directly to an epitope on the transcriptional regulator or it may bind to a tag on the regulator, such as a myc tag when used with an anti-Myc antibody (Santa Cruz

25   Biotechnology, sc-764). In yet another embodiment, a non-antibody agent with affinity for the transcriptional regulator or for a tag used to it is used in place of the antibody. For example, if the transcriptional regulator comprises an affinity tag, such as a six-histidine tag, complexes may be isolated by affinity chromatography to nickel-containing sepharose. Additional variations on ChIP methods may be found

30   in Kurdistani *et al.* Methods. 2003 31(1):90-5; O'Neill *et al.* Methods. 2003, 31(1):76-82; Spencer *et al.*, Methods. 2003;31(1):67-75; and Orlando *et al.* Methods 11: 205-214 (1997).

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In one embodiment of the methods described herein, DNA fragments from a control immunoprecipitation reaction are used in place of the isolated chromatin as a control. For example, an antibody that does not react with a transcription factor

5   being tested may be used in a chromatin IP procedure to isolate control chromatin, which can then be compared to the chromatin isolated using an antibody that does react with the transcriptional regulator. In preferred embodiments, the antibody that does not react with the transcription factor being tested also does not react with other transcriptional regulators or DNA binding proteins.

10

In one embodiment, labelled probes are generated from the chromatin fragments and optionally from control chromatin fragments. Labelled probes may be generated from template DNA using ligation-mediated polymerase chain reaction (LM-PCR), *e.g.*, see Current Protocols in Molecular Biology, Ausubel, F. M. *et al.*,

15   eds. 1991 and U.S. Application No. 2003/0143599, the teachings of which are incorporated herein by reference) in their entirety. In specific embodiments, LM-PCR comprises fluorescently labeling amplified DNA by including fluorescently-tagged nucleotides in the LM-PCR reaction. Generally, whole genome-based amplification methods (*e.g.*, substantially unbiased amplification methods) may be

20   used such as Multiple Displacement Amplification (MDA) (Hosono *et al, Genome Res.* 2003; 13(5):954-64), DOP (Telenius, *Genomics* 1992;13:718-725), primer extension preamplification or PEP (Zhang, *et al., Proc. Natl. Acad. Sci.1992;* 89:5847–5851), inter-Alu PCR in conjunction with labelled primers and/or nucleotides. However, in still other aspects, probes are labelled without a prior

25   amplification step.

In one embodiment of the methods described, labelled probes from control DNA fragments and the labelled probes are hybridized to a DNA microarray that includes experimental spots or features that represent all or a subset (*e.g.*, a

30   chromosome or chromosomes) of the genome. The fluorescent intensity of each experimental spot on the microarray from the amplified chromatin fragments relative to the amplified control chromatin indicates whether the protein of interest is

bound to the DNA region located at that particular spot. Hence, the methods described herein may be applied to the detection of protein-DNA interactions across an entire genome.

5   In some embodiments of the methods described herein, the labelled probes are hybridized to a DNA microarray to identify the regions of genomic DNA that were bound by the protein of interest. Microarrays, also called "biochips" or "arrays" are miniaturized devices typically with dimensions in the micrometer to millimeter range for performing chemical and biochemical reactions and are

10   particularly suited for embodiments of the invention. Arrays may be constructed via microelectronic and/or microfabrication using essentially any and all techniques known and available in the semiconductor industry and/or in the biochemistry industry, provided only that such techniques are amenable to and compatible with the deposition and screening of polynucleotide sequences. Microarrays are

15   particularly desirable for their virtues of high sample throughput and low cost for generating profiles and other data.

       DNA microarray and methods of analyzing data from microarrays are well-described in the art, including in DNA Microarrays: A Molecular Cloning Manual,

20   Ed by Bowtel and Sambrook (Cold Spring Harbor Laboratory Press, 2002); Microarrays for an Integrative Genomics by Kohana (MIT Press, 2002); A Biologist's Guide to Analysis of DNA Microarray Data, by Knudsen (Wiley, John & Sons, Incorporated, 2002); and DNA Microarrays: A Practical Approach, Vol. 205 by Schema (Oxford University Press, 1999); and Methods of Microarray Data

25   Analysis II, ed by Lin *et al.* (Kluwer Academic Publishers, 2002), hereby incorporated by reference in their entirety.

       In some embodiments, DNA microarray for use in the present invention may be constructed with spots comprising nucleic acid with promoter sequences. In some

30   embodiments of the methods described herein, the promoter region of a gene comprises from at least 700bp upstream to at least 200 bp downstream of the transcriptional start site of the gene. In some embodiments, the promoter region

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comprises at least about 30, 40, 50, or 60 nucleotides in length. In specific

embodiments, the promoter region of a gene as found on the spots of the microarray

comprises a sequence of at least 30 nucleotides whose sequence is identical to a

region stretching from 3 kb upstream to 1 kb downstream of the transcriptional start

5   site of said gene. Smaller probes (*e.g.*, about 30-200 nucleotides) spotted on the

microarray have the advantage that smaller corresponding genomic regions to which

the protein of interest is bound may be identified, but have the disadvantage that

more probes must be spotted onto the microarray to obtain the same coverage as

larger probes.

10

       In some embodiments, the sequence of the region of genomic DNA to which

the protein of interest is bound corresponds to the sequence of the nucleic acid on a

spot on the microarray to which the transcription factor is found to be bound. In

some embodiments where the spots on the microarray have overlapping sequences,

15   the sequences of the genome regions to the which the protein of interest binds may

be deduced to be shorter than the length of each of the spotted nucleic acids. For

example, if a protein of interest was found to bind to probes having sequences C-D-

E-F and E-F-G-H, wherein each letter represents stretches of for example 20-500bp,

and the protein of interest also failed to bind to probes having the sequences A-B-C-

20   D and G-H-I-J, then it could be deduced that the region to which the protein of

interest binds is E-F. Thus, in a preferred embodiment, the array comprises spotted

nucleic acids whose sequences are tiled *i.e.* share overlapping regions.

       In one embodiment of the methods described herein, identifying candidate

25   DNA-binding sites in the identified genomic regions comprises scanning the

sequence of the identified genomic regions to identify a sequence corresponding to

the DNA-sequence motif for the protein of interest. In an illustrative example, if

three genomic regions were identified, each of about 1 kb, and the DNA-sequence

motif for the protein of interest was GCANTGC, then each of the three 1kb

30   sequences would be scanned for a sequence that matches this motif, *i.e.* for any of

the subsequences GCAATGC, GCAGTGC, GCACTGC and GCATTGC. If such a

subsequence was identified in a genomic region, then that subsequence would be

determined to be a candidate DNA-binding site. Accordingly, a candidate DNA-binding site has a sequence identical to the DNA-sequence motif (*i.e.* it has an identical sequence to one of the subsequences of the DNA-sequence motif), and is present in an identified genomic region. An identified genomic region might contain

5   no candidate binding sites, it may contain one, or it may contain more than one.

Identification of candidate binding sites, having a sequence defined by the DNA-sequence motif in the genomic regions to which the protein of interest binds may be carried out through manual examination of the genomic regions. More

10   preferably however, software programs and algorithms known in the art are used to partially or fully automate the process. Programs that may be used in the present invention include, but are not limited to, the Ahb program (Rajewsky *et al.* (2002) *BMC Bioinformatics*, 3:30), the Clover program, (see Frith *et al.* (2004) *Nucleic Acids Res.*, 32, 1372–1381), and the MotifScanner program (Thijs *et al.* (2001),

15   Proceedings Recomb'2001, 305-312). In another embodiment, the Fuzznuc program is used to identify candidate binding sites in the set of genomic regions which correspond to the DNA-sequence motifs. Ffuzznuc is one program that is part of the EMBOSS suite of biological software tools distributed by the HGMP, UK (See Rice *et al.* (2000) EMBOSS: The European Molecular Biology Open Software Suite.

20   *Trends in Genetics*, Vol 16, No 6. pp.276-277). In yet another embodiment, the ROVER (Relative OVER-abundance of *cis*-elements) program is used to identify candidate binding sites (See Haverty *et al.* (2004) *Nucleic Acids Res.*, 32, 179–188). Additional approaches described in the literature may also be used (See Sharan *et al.* (2003). *Bioinformatics*, 19 (Suppl 1), I283–I291.; and Elkon *et al.* (2003) *Genome*

25   *Res.*, 13, 773–780.)

In some embodiments of the methods described herein, the DNA-sequence motif for a protein of interest is unknown or poorly defined. Accordingly, in some embodiments, the methods further comprises identifying a DNA-sequence motif for

30   the protein of interest from the sequences of the identified genomic DNA. In one embodiment, identifying a DNA-sequence motif generally comprises identifying one or more DNA sequences that are enriched in the regions of genomic DNA to which

the protein of interest is bound relative to a suitable control.

In a specific embodiment, the suitable control comprises a control set of genomic regions. The control set may comprise a set of genomic regions which are

5   not bound by the protein of interest in the cell.   In other embodiments, the control set comprises a set of randomly selected genomic regions. In yet another embodiment, the control set comprises randomized DNA sequences. In a preferred embodiment, the base composition of the randomized DNA sequences is substantially the same as base composition of either (i) the genome of the cell; (ii) the genomic regions to

10   which the protein of interest binds; or (iii) the average of promoter or other regulatory regions. For instance, if 60% and 40% of the basepairs in the genomic regions that are bound by the protein of interest are A-T and G-C basepairs respectively, then the randomized might contain this same proportion of basepairs. Alternatively, the suitable control may comprise an estimate of the frequency with

15   which a given motif is expected based on the frequency of base pairs in the motif, without the need to actually generate randomized sequences.

In another embodiment, the control set may comprise a set of genomic regions which is bound by a mutant form of the protein of interest, such as a mutant

20   form having deletions or substitutions in its DNA binding domain, whereas in other embodiments the control set comprises the set of genomic regions bound by a second protein, such as a transcription factor, a component of the basal transcription machinery, or a histone.

25   Any algorithm or software program known to one skilled in the art to identify DNA-sequence motifs may be used to identify statistically-enriched sets of DNA-sequence motifs from the set of identified genomic regions. In some embodiments, the DNA sequence motif is identified using one or more of the following programs or algorithms: Gibbs Sampler (Rajewsky *et al.* (2002) BMC

30   Bioinformatics, 3:30; Lawrence *et al.* (1993) *Science*, 262, 208–214.); R'MES programs (Schbath S, (1997) *J. Comp. Biol.*, 4, 189-192), the Verbumculus program (Apostolico *et al.*, (2000) Journal of Computational Biology, vol.7, no.1/2,;

Apostolico *et al.* (2004) *Journal of Computer and Science Technology*, vol.19, no.1,
pp.22-41), the YMF program (Sinha *et al.*, (2002) *Nucleic Acids Research*, vol. 30,
no. 24, 5549-5560, and Sinha *et al.* (2000) *Eighth International Conference on
Intelligent Systems for Molecular Biology*, San Diego, CA, 344-354); the AlignACE

5   (Aligns Nucleic Acid Conserved Elements) (Hughes *et al* (2000), Journal of
Molecular Biology; 296(5):1205-14, and Roth *et al*, (1998) *Nature* Biotechnology,
16(10):939-45, 1998), the BioProspector program (Liu X *et al* (2001) *Pac. Symp.
Biocomput.*,127-38, the MEME program (Bailey *et al* (1994) *Proceedings of the
Second International Conference on Intelligent Systems for Molecular Biology*, 28-

10   36, AAAI Press); Motif Sampler (Thijs G (2001) *et al. Bioinformatics*, 17(12),1113-
1122, and Thijs G *et al.* Journal of Computational Biology (special issue
Recomb'2001), 9(2), 447-464, 2002), and SUPERPOSITION (Shinozaki D *et al.*,
(2003) *Bioinformatics*;19 Suppl 2:II206-II214).

15   In one specific embodiment, the Motif Discovery scan (MDscan) program is
used to identify DNA-sequence motifs from the set of identified genomic regions
(Liu XS*et al.* (2002) *Nat. Biotechnol.*, 20(8):835-9). In another specific
embodiment, the Mogul program, which incorporates multiple algorithms, is used to
identify the DNA-sequence motifs (Rust *et al. Int. Conf. on Systems Biology

20   (ISMB2003),* 2003). Additional algorithms have also been described (see Bailey *et
al.* (1994) *Proc. Int. Conf. Intell. Syst. Mol. Biol.*, 2, 28—36. In some embodiments,
more than one algorithm is used to identify the DNA-sequence motif.

In another embodiment, the validity of the DNA-sequence motif that is
25   identified is experimentally tested using such approaches as the one-hybrid system
or *in vitro* DNA-binding site selection methods. (See Blackwell TK (1995) *Methods
Enzymol.*;254:604-18; Blackwell *et al.* (1990) *Science.* 250(4984):1149-51;
Blackwell *et al.* (1990) *Science.* 250(4984):1104-10). Additional methods are
disclosed in U.S. Patent Application No. 2004/0115794, hereby incorporated by
30   reference in its entirety.

In another embodiment, at least one of the following motif-finding programs

is used: AlignACE (Roth, F. P., Hughes, J. D., Estep, P. W. & Church, G. M.
Finding DNA regulatory motifs within unaligned noncoding sequences clustered by
whole-genome mRNA quantitation. *Nat Biotechnol* 16, 939-45 (1998)), MEME
(Bailey, T. L. & Elkan, C. The value of prior knowledge in discovering motifs with

5   MEME. *Proc Int Conf Intell Syst Mol Biol* 3, 21-9 (1995)), MDscan (Liu, X. S.,
Brutlag, D. L. & Liu, J. S. An algorithm for finding protein-DNA-binding sites with
applications to chromatin-immunoprecipitation microarray experiments. *Nat
Biotechnol* 20, 835-9 (2002)), the conservation-based method described in Kellis *et
al.* (Kellis *et al.* Sequencing and comparison of yeast species to identify genes and

10   regulatory elements. *Nature* 423, 241-54 (2003)), herein referred to as the "Kellis
Method", and a new conservation-based method called CONVERGE.

One embodiment of the methods described herein for identifying a set of
biologically-active DNA-binding sites for a protein of interest comprises

15   determining if candidate DNA-binding sites are conserved in one or more species
different from that of the cell. In a specific embodiment, the method comprises
determining if the candidate DNA-binding sites are conserved in the equivalent
genomic regions in one or more species. In one embodiment, the different species
are classified under the same phylogenetic class as the cell. In another embodiment,

20   the different species are classified under the same order as the cell. In a preferred
embodiment, the different species are classified under the same genus as the cell. In
another embodiment, the percent sequence identity between the ribosomal RNA of
the cell and that of the different species is at least 70%, 75%, 80%, 85%, 90%, 91%,
92%, 93%, 94%, 95%, 96%, 97%, 97.5%, 98%, 99%, 99.5%, 99.8% or 99.9%. In

25   another embodiment, the percent sequence identity between the mtDNA of the cell
and that of the different species is at least 70%, 75%, 80%, 85%, 90%, 91%, 92%,
93%, 94%, 95%, 96%, 97%, 97.5%, 98%, 99%, 99.5%, 99.8% or 99.9%. In another
embodiment, the average percent sequence identity between the coding regions of
the cell and that of the different species is at least 70%, 75%, 80%, 85%, 90%, 91%,

30   92%, 93%, 94%, 95%, 96%, 97%, 97.5%, 98%, 99%, 99.5%, 99.8% or 99.9%.

In one embodiment, the candidate DNA-binding site is said to be a

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biologically-active DNA-binding site if a sequence matching the DNA-binding motif is present in the equivalent genomic region. In a specific embodiment, the candidate DNA-binding site is said to be a biologically-active DNA-binding site if a sequence that matches the DNA-binding motif is present in the equivalent genomic

5   region in at least one different species. In one embodiment, two regions of genomic DNA are said to be equivalent if they are orthologous sequences, such as those containing orthologous genes. The term "orthologous genes" refers to gene loci in different species that are sufficiently similar to each other in their nucleotide sequences to suggest that they originated from a common ancestral gene.

10   Orthologous genes arise when a lineage splits into two species, rather than when a gene is duplicated within a genome. Proteins that are orthologs are encoded by genes of two different species, wherein the genes are said to be orthologous. In a related embodiment, two regions are said to be equivalent if they comprise orthologous gene sequences.

15

      In one embodiment, the identification of equivalent, or orthologous, genomic regions may be performed using DNA-alignment programs to compare the identified genomic regions to genomic sequences from another species. In one embodiment, a sequence of at least 50, 100, 200, 400, 600, 800, 1000, 1500, 3000, 5000 or 10000

20   bases flanking the candidate binding site is used to search for an equivalent genomic regions in the genomic sequence of other species. Programs and algorithms for the alignment and comparison of DNA sequences are well known in the art. In one embodiment, the DNA Block Aligner (DBA) program, part of the GeneWise software set, is used to compare the identified genomic sequence to genomic DNA

25   from another species (See Ewan *et al.* (2004) GeneWise and Genomewise. *Genome Research* 14: 988-995). Methods for identifying evolutionarily conserved sequences have been described in U.S. Patent Publication Nos. 2003/0017474 and 2003/0119015, the entire contents of which are herein incorporated by reference. Other programs that may be used include the Mauve program (Darling *et al.*(2004)

30   *Genome Res*;14(7):1394-403), the OrthoMCL program (Li *et al.* (2003) *Genome Res.*;13(9):2178-89). See also Thomas *et al.* (2003) *Nature* 14;424(6950):788-93.

Nucleic acid sequence homologies between genomic regions may be evaluated using any of the variety of sequence comparison algorithms and programs known in the art. Such algorithms and programs include, but are by no means limited to, TBLASTN, BLASTP, FASTA, TFASTA, and CLUSTALW (Pearson

5   and Lipman, 1988, Proc. Natl. Acad. Sci. USA 85(8):2444-2448; Altschul et al., 1990, J. Mol. Biol. 215(3):403-410; Thompson et al., 1994, Nucleic Acids Res. 22(2):4673-4680; Higgins et al., 1996, Methods Enzymol. 266:383-402; Altschul et al., 1990, J. Mol. Biol. 215(3):403-410; Altschul et al., 1993, Nature Genetics 3:266-272). In a specific embodiment, nucleic acid sequence homologies are

10   evaluated using the Basic Local Alignment Search Tool ("BLAST") which is well known in the art (see, e.g., Karlin and Altschul, 1990, Proc. Natl. Acad Sci. USA 87:2267-2268; Altschul et al., 1990, J. Mol. Biol. 215:403-410; Altschul et al., 1993, Nature Genetics 3:266-272; Altschul et al., 1997, Nuc. Acids Res. 25:3389-3402).

15

The BLAST programs identify homologous sequences by identifying similar segments, which are referred to herein as "high-scoring segment pairs," between a query amino or nucleic acid sequence and a test sequence which is preferably obtained from a protein or nucleic acid sequence database. High-scoring segment

20   pairs are preferably identified (i.e., aligned) by means of a scoring matrix, many of which are known in the art. The scoring matrix used may be the BLOSUM62 matrix (Gonnet et al., 1992, Science 256:1443-1445; Henikoff and Henikoff, 1993, Proteins 17:49-61). The PAM or PAM250 matrices may also be used (see, e.g., Schwartz and Dayhoff, eds., 1978, Matrices for Detecting Distance Relationships: Atlas of Protein

25   Sequence and Structure, Washington: National Biomedical Research Foundation). The BLAST programs evaluate the statistical significance of all high-scoring segment pairs identified, and preferably selects those segments which satisfy a user-specified threshold of significance, such as a user-specified percent homology. In one embodiment, the statistical significance of a high-scoring segment pair is

30   evaluated using the statistical significance formula of Karlin (see, e.g., Karlin and Altschul, 1990, Proc. Natl. Acad. Sci. USA 87:2267-2268). The parameters used with the above algorithms may be adapted depending on the sequence length and

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degree of homology studied. In some embodiments, the parameters may be the default parameters used by the algorithms in the absence of instructions from the user.

5   The comparison of sequences and determination of percent identity and similarity between two sequences can be accomplished using a mathematical algorithm. (Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data,
10   Part 1, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991). In one preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (J Mol.
15   Biol. (48):444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blossom 62 matrix or a *PAM250* matrix. In yet another embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (Devereux, J., *et al.*, Nucleic Acids Res.
20   12(1):387 (1984)) (available at http://www.gcg.com), using a NWSgapdna-CMP matrix. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Myers and W. Miller (CABIOS, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0).
25

          In a specific embodiment, a candidate DNA-binding site is a conserved DNA-binding site if a DNA-binding site corresponding to the DNA-sequence motif of the protein of interest is present within a genomic region in another species that is equivalent to a window of less than 2kb, 1.5kb, 1kb, 800bp, 600bp, 500bp, 400bp,
30   300bp, 200bp, 150bp, 100bp, 80bp, 60bp or 50bp flanking the candidate DNA-binding sites. For example, in an illustrative non-limiting example, in which a candidate DNA-binding site of sequence GGGACCC lies 550bp upstream of the

transcriptional start site of gene X in the cell, where the window is 300bp and the DNA-sequence motif for the protein of interest is GGG$\underline{N}$CCC, then the candidate DNA-binding site would be conserved if any of the sequences GGG$\underline{A}$CCC, GGG$\underline{T}$CCC, GGG$\underline{G}$CCC or GGG$\underline{C}$CCC were found in the region of the other

5   species equivalent to from 700-400bp from the transcriptional start site of gene X, since they all match the DNA-sequence motif. Depending on the sequence identity between the two species in the region preceding the window and the transcriptional start site, the region in the second species that is equivalent to the 700-400bp window from the cell may be shifted relative to the transcriptional start site, such

10 that the equivalent positions may be from 690-390bp if, for instance, the second species had a 10bp deletion immediately preceding the transcriptional start site. One skilled in the art would be able to ascertain any such shifts based on the sequence alignments between the two regions.

15   In a specific embodiment, a candidate DNA-binding site is said to be a conserved DNA-binding site if a DNA-binding site corresponding to the DNA-sequence motif of the protein of interest is present within the promoter of the orthologous gene in another species. In a specific embodiment, a candidate DNA-binding site within the promoter of a gene is said to be a conserved DNA-binding

20 site if a DNA-binding site corresponding to the DNA-sequence motif of the protein of interest is present in the promoter of the orthologous gene in another species, wherein the promoter region comprises from about 5kb, 4kb, 3kb, 2.5kb, 2kb, 1.5kb, 1kb, 900bp, 800bp or 700bp 5' to the transcriptional start site to about 50bp, 100bp, 150bp, 200bp, 250bp, 300bp, 400bp, 500bp, 600bp, 800bp or 1000bp 3' to the

25 transcriptional start site. In another embodiment, the promoter region consists of from about5kb, 4kb, 3kb, 2.5kb, 2kb, 1.5kb, 1kb, 900bp, 800bp or 700bp 5' to the transcriptional start site to about 50bp, 100bp, 150bp, 200bp, 250bp, 300bp, 400bp, 500bp, 600bp, 800bp or 1000bp 3' to the transcriptional start site. In another embodiment, some or all of the promoter regions may have been experimentally

30 defined, and as a result, the size of the promoter regions between different genes may differ.

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IV. Additional Methods

Another aspect of the invention provides methods of identifying agents which alter the set of biologically-active DNA-binding sites for a protein of interest in the genome of a cell. One specific aspect provides a method of identifying an

5   agent which alters the set of biologically-active DNA-binding sites for a protein of interest in the genome of a cell, the method comprising (i) contacting an experimental cell with a candidate agent; (ii) identifying a set of biologically-active DNA-binding sites for a protein of interest in the genome of the cell of step (i) according to any of the methods described herein, thereby generating an

10   experimental set of biologically-active DNA-binding sites; (iii) comparing (1) the experimental set of biologically-active DNA-binding sites to (2) a control set of biologically-active DNA-binding sites for the protein of interest; wherein a candidate agent is identified if the experimental set and the control set differ.

15   In one embodiment of the methods for identifying an agent, the control set of biologically-active DNA-binding sites to which the experimental set of biologically-active DNA-binding sites is a set of biologically-active DNA-binding sites for the protein of interest in a control cell that has not been contacted with an agent. In one embodiment, the control set of biologically-active DNA-binding sites is the set of

20   biologically-active DNA-binding sites for the protein of interest in a control cell that (a) has been contacted with a different dosage of the agent; (b) has been contacted with a different agent; or (c) a combination thereof. Alternatively, the control set may be derived from the cell or a group of cells prior to contacting with the agent. In some embodiments, the methods comprise contacting a population of cells with the

25   agent, rather than a single cell, with subsequent steps using the population of cells or a subpopulation.

In preferred embodiments, experimental conditions, other than contacting the experimental cell with the agent, are substantially the same between the

30   experimental cell and the control cell, such as the genotype of the cell, the growth conditions of the cell, conditions for the isolation of chromatin, immunoprecipitation conditions, *etc*. In one embodiment, the experimental cell is contacted with the

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agent *in vitro*, whereas in other embodiments the cell is contacted *in vivo*. A cell may be contacted *in vivo* with the agent, for example, by administering the agent to an organism which comprises the cell.

5   In some embodiments, a cell is contacted with an agent by genetically expressing the agent in the cell, such as by introducing a transgene in the experimental cell which encodes an agent, such as an RNA or a polypeptide agent. The transgene may encode any protein, such as a transcriptional regulator or a protein that regulates the activity of transcriptional regulators, such as a kinase and 10   phosphatase. The transgene may also encode an inhibitory RNA, such as a hairpin RNA, so that the function of the gene to which the hairpin RNA is directed may be knocked down. In some embodiments, the transgene is associated with a disease state.

15   In some embodiments, the candidate agent is identified if the experimental set and the control set differ by at least one biologically-active DNA-binding site which is present in one set but not in the other. In other embodiments, the candidate agent is identified if at least 2, 3, 4, 5, 10, 20 or 30 of the biologically-active DNA-binding sites are differentially present in one set relative to the other. In another 20   embodiment, the candidate agent is identified if at least 0.5%, 1%, 2%, 3%, 4% or 5% or 10% of the biologically-active DNA-binding sites are differentially present in one set relative to the other.

       A related aspect of the invention provides methods of identifying conditions 25   in which a protein of interest differentially binds to the genome of a cell. One specific aspect of the invention provides a method of identifying two sets of conditions in which a protein of interest differentially binds to the genome of a cell, the method comprising: (i) identifying a first set of biologically-active DNA-binding sites for the protein of interest in the genome of a cell according to any of the 30   methods described herein, wherein the cell is exposed to a first set of conditions; (ii) identifying a second set of biologically-active DNA-binding sites for the protein of interest in the genome of a cell according to any of the methods described herein,

wherein the cell is exposed to a second set of conditions; and (iii) comparing the first set of biologically-active DNA-binding sites to the second set of biologically-active DNA-binding sites and determining if the two sets differ.

5   In one embodiment, the set of conditions comprise a set of environmental conditions. The condition may be a physical condition of the environment in which the cell resides, a chemical condition of the environment, and/or a biological condition of the site. Exposure may be for any suitable time. The exposure may be continuous, transient, periodic, sporadic, *etc.* Physical conditions include any

10   physical state of the sample in which the cell resides. The physical state may be the temperature or pressure of the sample, or an amount or quality of light (electromagnetic radiation) at the site. Alternatively, or in addition, the physical state may relate to an electric field, magnetic field, and/or particle radiation at the site, among others. Chemical conditions include any chemical aspect of the fluid in

15   which the sample populations are disposed. The chemical aspect may relate to the pH, ionic strength, and/or fluid composition such as gas concentrations, among others. If the cell is in an organism, the set of conditions may include diet, physical activity, sexual activity, stress levels or awareness states such as awake vs. sleeping states, and a diseased state, among others.

20

          In another embodiment, the set of conditions comprises a set of biological conditions. Biological conditions include any biological aspect of the shared fluid volume in which cell is disposed, such as nutrients. The biological conditions may also include the presence or absence of growth factors, chemokines or cytokines.

25   The biological aspects may include the presence, absence, concentration, activity, or type of additional cells.

          A related aspect of the invention also provides a method of identifying two cell genotypes in which a protein of interest differentially binds to the genome of a

30   cell, the method comprising: (i) identifying a first set of biologically-active DNA-binding sites for the protein of interest in the genome of a cell of a first genotype; (ii) identifying a second set of biologically-active DNA-binding sites for the protein of

interest in the genome of a cell of a second genotype; (iii) comparing the first set of biologically-active DNA-binding sites to the second set of biologically-active DNA-binding sites and determining if the two sets differ.

5   In one embodiment, the genotypes of the cells differs by the genotype of the gene encoding the protein of interest. In other embodiments, the cells differ in the genotypes of multiple genes, one of which may be the protein of interest.

          Another aspect of the invention provides a method of identifying at least one
10   pathway that is regulated by a protein of interest in a cell. One specific aspect of the invention provides a method of identifying a pathway that is transcriptionally regulated by a protein of interest in a cell, the method comprising (i) identifying a set of biologically-active DNA-binding sites for a protein of interest in the genome of the cell according to any of the methods described herein; and (ii) identifying at
15   least two candidate genes likely to be regulated by binding of the protein of interest to the set of biologically-active DNA-binding sites identified in (i); wherein a pathway is identified if at least two candidate genes are members of the same pathway.

20   As used herein, the term "pathway" is intended to mean a set of system components involved in two or more sequential molecular interactions that result in the production of a product or activity. A pathway can produce a variety of products or activities that can include, for example, intermolecular interactions, changes in expression of a nucleic acid or polypeptide, the formation or dissociation of a
25   complex between two or more molecules, accumulation or destruction of a metabolic product, activation or deactivation of an enzyme or binding activity. Thus, the term "pathway" includes a variety of pathway types, such as, for example, a biochemical pathway, a gene expression pathway and a regulatory pathway. Similarly, a pathway can include a combination of these exemplary pathway types.
30
          In one embodiment, the pathway is a biochemical pathway. A biochemical pathway can include, for example, enzymatic pathways that result in conversion of

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one compound to another, such as in metabolism, and signal transduction pathways
that result in alterations of enzyme activity, polypeptide structure, and polypeptide
functional activity. Specific examples of biochemical pathways include the pathway
by which galactose is converted into glucose-6-phosphate and the pathway by which

5   a photon of light received by the photoreceptor rhodopsin results in the production
of cyclic AMP. Numerous other biochemical pathways exist and are well known to
those skilled in the art.

In some embodiments, the biochemical pathway is a carbohydrate

10   metabolism pathway, which in a specific embodiment is selected from the group
consisting of glycolysis / gluconeogenesis, citrate cycle (TCA cycle), pentose
phosphate pathway, pentose and glucuronate interconversions, fructose and mannose
metabolism, galactose metabolism, Ascorbate and aldarate metabolism, starch and
sucrose metabolism, amino sugars metabolism, nucleotide sugars metabolism,

15   pyruvate metabolism, glyoxylate and dicarboxylate metabolism, propionate
metabolism, butanoate metabolism, C_5-branched dibasic acid metabolism, inositol
metabolism and inositol phosphate metabolism.

In some embodiments, the biochemical pathway is an energy metabolism

20   pathway, which in a specific embodiment is selected from the group consisting of
oxidative phosphorylation, ATP synthesis, photosynthesis, carbon fixation,
reductive carboxylate cycle (CO_2 fixation), methane metabolism, nitrogen
metabolism and sulfur metabolism.

25   In some embodiments, the biochemical pathway is a lipid metabolism
pathway, which in a specific embodiment is selected from the group consisting of
fatty acid biosynthesis (path 1), fatty acid biosynthesis (path 2), fatty acid
metabolism, synthesis and degradation of ketone bodies, biosynthesis of steroids,
bile acid biosynthesis, C21-steroid hormone metabolism, androgen and estrogen

30   metabolism, glycerolipid metabolism, phospholipid degradation, prostaglandin and
leukotriene metabolism.

In some embodiments, the biochemical pathway is a nucleotide metabolism pathway, which in a specific embodiment is selected from the group consisting of purine metabolism and pyrimidine metabolism.

5   In some embodiments, the biochemical pathway is an amino acid metabolism pathway, which in a specific embodiment is selected from the group consisting of glutamate metabolism, alanine and aspartate metabolism, glycine, serine and threonine metabolism, methionine metabolism, cysteine metabolism, valine, leucine and isoleucine degradation, valine, leucine and isoleucine biosynthesis, lysine

10   biosynthesis, lysine degradation, arginine and proline metabolism, histidine metabolism, tyrosine metabolism, phenylalanine metabolism, tryptophan metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, urea cycle, beta-Alanine metabolism, taurine and hypotaurine metabolism, aminophosphonate metabolism, selenoamino acid metabolism, cyanoamino acid metabolism, D-

15   glutamine and D-glutamate metabolism, D-arginine and D-ornithine metabolism, D-alanine metabolism and glutathione metabolism.

In some embodiments, the biochemical pathway is a glycan biosynthesis and metabolism pathway, which in a specific embodiment is selected from the group

20   consisting of N-glycans biosynthesis, N-glycan degradation, O-glycans biosynthesis, chondroitin / heparan sulfate biosynthesis, keratan sulfate biosynthesis, glycosaminoglycan degradation, lipopolysaccharide biosynthesis, clycosylphosphatidylinositol(GPI)-anchor biosynthesis, peptidoglycan biosynthesis, glycosphingolipid metabolism, blood group glycolipid biosynthesis - lactoseries,

25   blood group glycolipid biosynthesis - neo-lactoseries, globoside metabolism and ganglioside biosynthesis.

In some embodiments, the biochemical pathway is a biosynthesis of Polyketides and Nonribosomal Peptides pathway, which in a specific embodiment is

30   selected from the group consisting of Type I polyketide structures, biosynthesis of 12-, 14- and 16-membered macrolides, biosynthesis of ansamycins, polyketide sugar unit biosynthesis, nonribosomal peptide structures, and siderophore group

nonribosomal peptide biosynthesis.

In some embodiments, the biochemical pathway is a metabolism of cofactors and vitamins pathway, which in a specific embodiment is selected from the group
5   consisting of Thiamine metabolism, Riboflavin metabolism, Vitamin B6 metabolism, Nicotinate and nicotinamide metabolism, Pantothenate and CoA biosynthesis, Biotin metabolism, Folate biosynthesis, One carbon pool by folate, Retinol metabolism, Porphyrin and chlorophyll metabolism and Ubiquinone biosynthesis.
10

In some embodiments, the biochemical pathway is a biosynthesis of secondary metabolites pathway, which in a specific embodiment is selected from the group consisting of terpenoid biosynthesis, diterpenoid biosynthesis, monoterpenoid biosynthesis, limonene and pinene degradation, indole and ipecac alkaloid
15   biosynthesis, flavonoids, stilbene and lignin biosynthesis, alkaloid biosynthesis I, alkaloid biosynthesis II, penicillins and cephalosporins biosynthesis, beta-lactam resistance, streptomycin biosynthesis, tetracycline biosynthesis, clavulanic acid biosynthesis and puromycin biosynthesis.

20   In one embodiment, the pathway is a gene expression pathway. A gene expression pathway can include, for example, molecules which induce, enhance or repress expression of a particular gene. A gene expression pathway can therefore include polypeptides that function as repressors and transcription factors that bind to specific DNA sequences in a promoter or other regulatory region of the one or more
25   regulated genes. An example of a gene expression pathway is the induction of cell cycle gene expression in response to a growth stimulus.

In one embodiment, the pathway is a regulatory pathway. A regulatory pathway can include, for example, a pathway that controls a cellular function under
30   a specific condition. A regulatory pathway controls a cellular function by, for example, altering the activity of a system component or the activity of a biochemical, gene expression or other type of pathway. Alterations in activity

include, for example, inducing a change in the expression, activity, or physical interactions of a pathway component under a specific condition. Specific examples of regulatory pathways include a pathway that activates a cellular function in response to an environmental stimulus of a biochemical system, such as the

5   inhibition of cell differentiation in response to the presence of a cell growth signal and the activation of galactose import and catalysis in response to the presence of galactose and the absence of repressing sugars. The term "component" when used in reference to a network or pathway is intended to mean a molecular constituent of the biochemical system, network or pathway, such as, for example, a polypeptide,

10   nucleic acid, other macromolecule or other biological molecule.

In one embodiment, the pathway is a signaling pathway. Signaling pathways include MAPK signaling pathways, Wnt signaling pathways, TGF-beta signaling pathways, toll-like receptor signaling pathways, Jak-STAT signaling pathways,

15   second messenger signaling pathways and phosphatidylinositol signaling pathways.

One embodiment of the methods described herein for identifying pathways comprises identifying candidate genes likely to be regulated by the protein of interest. In one embodiment, a candidate gene is likely regulated by the protein of

20   interest if the promoter for the candidate gene comprises at least one of the identified biologically-active DNA-binding sites for the protein of interest. In a specific embodiment, the promoter region of a candidate gene comprises from about 3kb 5' to 1kb 3' of the transcription initiation site. In another specific embodiment, the promoter region of a candidate gene comprises from about 2 kb 5' to 0.5 kb 3' of the

25   transcription initiation site. In another embodiment, a candidate gene is likely regulated by the protein of interest if a regulatory element for the candidate gene comprises at least one of the identified biologically-active DNA-binding sites. In one embodiment, the regulatory element is selected from the group consisting of promoter and enhancer regions.

30

In one embodiment, a candidate gene is likely regulated by the protein of interest if at least one of the identified biologically-active DNA-binding sites is

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located within about 3kb 5' to 1kb 3' of the transcription initiation site for the gene. If a gene has more than one transcription initiation site, then in one embodiment the candidate gene is likely regulated by the protein of interest if at least one of the identified biologically-active DNA-binding sites is located within about 3kb 5' to

5   1kb 3' of at least one of the transcription initiation sites for the gene. In a specific embodiment, at least one of the identified biologically-active DNA-binding sites is located within about 2 kb 5' to 0.5 kb 3' of at least one of the transcription initiation sites for the gene.

10   In another embodiment, a candidate gene is likely regulated by the protein of interest if at least one of the identified biologically-active DNA-binding sites is located within about 4 kb 5' to 1 kb 3' of the initiator methionine codon for the gene of interest, or where multiple gene products exist which differ in the initiator methionine use, at least one of the identified biologically-active DNA-binding sites

15   is located within about 4 kb 5' to 1 kb 3 or one of the initiator methionine.

Candidate genes may be assigned to pathways by one skilled in the art, for example, by consulting any of several databases which describe the function of genes and their classification into pathways and/or by consulting the literature (See

20   also Biochemical Pathways: An Atlas of Biochemistry and Molecular Biology. Gerhard Michal (Editor) Wiley, John & Sons, Incorporated, (1998); Biochemistry of Signal Transduction and Regulation, Gerhard Krauss, Wiley, John & Sons, Incorporated, (2003); Signal Transduction. Bastien D. Gomperts, Academic Press, Incorporated (2003)). Databases which may be used include, but are not limited to,

25   http://www.genome.jp/kegg/kegg4.html; Pubmed, OMIM and Entrez at http://www.ncbi.nih.gov; the Swiss-Prot database at http://www.expasy.org/.

In one preferred embodiment, a pathway to which a gene has been assigned is identified using the Biomolecular Interaction Network Database (BIND) at

30   http://www.blueprint.org/bind/, and more preferably at http://www.blueprint.org /bind/ search/bindsearch.html (See also Bader GD, Betel D, Hogue CW. (2003) BIND: the Biomolecular Interaction Network Database. *Nucleic Acids Res.*

31(1):248-50; and Bader GD, Hogue CW. (2003) An automated method for finding molecular complexes in large protein interaction networks. *BMC Bioinformatics.* 4(1)). One feature of the BIMD database lists the pathways to which a query gene has been assigned, thereby allowing the identification of the pathways to which a

5   gene is assigned. Furthermore, U.S. Patent Publication No. 2003/0100996 describes methods for establishing a pathway database and performing pathway searches which may be used to facilitate the identification of pathways and the classification of genes into pathways.

10   Another aspect of the invention provides methods of identifying the mechanisms underlying gene regulation. The invention provides methods of identifying the biochemical changes for a protein of interest which correlate with its differential binding to the genome of a cell. One specific aspect of the invention provides a method of identifying a property of a gene product of a gene of interest

15   that correlates with the binding activity of a polypeptide encoded by the gene of interest to the genome of a cell, the method comprising (i) identifying two sets of conditions in which a protein of interest differentially binds to the genome of the cell according to any of the methods described herein; (ii) determining a property of a gene product of the gene of interest in (a) a cell exposed to a first set of conditions;

20   and in (b) a cell exposed to a second set of conditions; and (iii) determining if at least one property of the gene product differs in the two cells of step (ii), thereby identifying a property that correlates with the binding activity of a gene of interest to the genome of a cell.

25   Another aspect of the invention provides a method of identifying a property of a gene product of a gene of interest that correlates with the binding activity of a polypeptide encoded by the gene of interest to the genome of a cell, the method comprising (i) identifying an agent which alters the set of biologically-active DNA-binding sites for a protein of interest in the genome of a cell according to any of the

30   methods described herein; (ii) determining a property of a gene product of the gene of interest in (a) a cell contacted with the agent; and in (b) a cell not contacted with the agent; and (iii) determining if at least one property of the gene product differs in

the two cells of step (ii), thereby identifying a property that correlates with the binding activity of a gene of interest to the genome of a cell.

5 A related aspect of the invention provides a method of identifying a property of the gene product of a gene of interest that correlates with the binding activity of a polypeptide encoded by the gene of interest to the genome of a cell, the method comprising (i) identifying an agent which alters the set of biologically-active DNA-binding sites for a protein of interest in the genome of a cell according to any of the methods described herein; (ii) determining a property of a gene product of the gene
10 of interest in (a) a cell contacted with dosage of the agent; and in (b) a cell contacted with a different dosage of the agent; and (iii) determining if at least one property of the gene product differs in the two cells of step (ii), thereby identifying a property that correlates with the binding activity of a gene of interest to the genome of a cell. A different dosage of the agent may comprise, for example, exposing the cell to a
15 different concentration of the agent of for a different duration.

In one embodiment of the methods described herein for identifying a property of a gene product, the expression product is an mRNA. In another embodiment, the expression product is a polypeptide.
20

In one embodiment of the methods described herein for identifying a property of a gene product, the property of the gene product is selected from the group consisting of a post-translational modification of the gene product, expression level of the gene product, enzymatic activity of the gene product, oligomeric state
25 and intracellular localization of the gene product. As used herein, "post-translational modification" refers to addition of a peptidic or non-peptidic moiety to a protein that cannot be considered as the elongation of the peptidic chain of the protein. Exemplary post-translational protein modification include phosphorylation, acetylation, methylation, ADP-ribosylation, ubiquitination, glycosylation,
30 carbonylation, sumoylation, biotinylation or addition of a polypeptide side chain or of a hydrophobic group. In one specific embodiment, the phosphorylation to be assessed is phosphorylation on tyrosine, serine, threonine or histidine residue. In

another specific embodiment, the addition of a polypeptide side chain to be assessed is the addition of ubiquitin. Ubiquitination involves the covalent attachment of ubiquitin, an evolutionary highly conserved 76-amino acid polypeptide which is abundantly present in all eukaryotic cells to the 1-amino group of one or more lysine

5   side chains of target proteins. In still another specific embodiment, the addition of a hydrophobic group to be assessed is the addition of a fatty acid, *e.g.*, myristate or palmitate, addition of an isoprenoid, *e.g.*, farnesyl or genranylgenranyl, or addition of a glycosyl-phosphatidyl inositol anchor, *e.g.*, a carbohydrate group comprises glycosyl.

10

Phosphorylation can include phosphorylation of a tyrosine, serine, threonine or histidine. Antibodies that can be used to detect these modifications can include phosphotyrosine-specific antibody, phosphoserine-specific antibody, phosphoserine-specific antibody, and phospho-threonine-proline antibody, for

15   example. Antibodies that can be used to detect these modifications also include an antibody specific to a phosphorylated residue of a protein such as phosphorylated c-Jun at Ser 73. Acetylation can be detected by using an acetylated-lysine antibody. Methylation specific antibodies can be used to detect proteins having a methylation on one or more amino acids. ADP-ribosylation specific antibodies can be used to

20   detect proteins having an ADP ribosylation modification. An example of addition of a polypeptide chain is ubiquitination. Detection of ubiquitination on a target protein can be made using an ubiquitin-specific antibody or polyubiquitin-specific antibody for example.

25   Any general method known to one skilled in the art may be applied to determine the expression level of a gene product, such as determining mRNA levels or protein levels. Levels of mRNA may be determined, for example, using such techniques as Northern Blots, reverse-transcriptase polymerase chain reaction (RT-PCR), RNA protection assays or a DNA microarray comprising probes capable of

30   hybridizing to at least a portion of the mRNA. Likewise, protein levels may be quantitated using techniques well-known in the art, such as western blotting, immuno-sandwich assays, ELISA assays, or any other immunological technique.

Techniques for quantitating nucleic acids and proteins may be found, for example, in Molecular Cloning: A Laboratory Manual, 3rd Ed., ed. by Sambrook and Russell (Cold Spring Harbor Laboratory Press: 2001); and in Current Protocols in Cell Biology, ed. by Bonifacino, Dasso, Lippincott-Schwartz, Harford, and Yamada,

5   John Wiley and Sons, Inc., New York, 1999, hereby incorporated by reference in their entirety.

The intracellular localization of a protein may be determined by immunocytochemistry. Alternatively, organelles from the cells may be purified and

10   the presence of the polypeptide in different organelle preparations determined. Alternatively, the protein of interest may be fused to a fluorescent protein to allow *in vivo* live imaging of the intracellular localization of the protein. Exemplary fluorescent proteins include the green fluorescent protein (GFP), DsRed, zFP538, mRFP1, BFP, CFP, YFP, mutants thereof, or functionally-active fragments thereof.

15   GFP is described in U.S. Pat. No. 5,491,084, while zFP538 is described in Zagranichny *et al.* Biochemistry. 2004; 43(16):4764-72. Subcellular compartments include but are not limited to, nucleus, endoplasmic reticulum (ER), Golgi apparatus, coated pits, mitochondria, endosomes, and cytoplasm. The intracellular localization of an mRNA molecular may also be determined using in-situ

20   hybridization techniques on fixed cell samples.

In one embodiment where the gene product is an enzyme, the enzymatic activity of the gene product is the property of the gene product. Enzymatic activity of a gene product may be determined using standard assays for

25   that particular type of enzyme (see for example Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis, by Robert Allen Copeland; Wiley, John & Sons, Incorporated, 2000; and Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems; by Irwin H. Segel; Wiley, John & Sons, Incorporated (1994)).

30

In one embodiment of the methods described herein for identifying a property of a gene product, the property of the gene product is the oligomeric state

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of the polypeptide gene product. Oligomeric state refers to whether the protein of interest exists as a monomer, dimer, trimer, hexamer or other type of multimeric state. The oligomeric state of a protein of interest may be determined, for example, using non-denaturing polyacrylamide gel electrophoresis (PAGE) or gel filtration

5   chromatography to assess the size of a complex containing the proteins of interest.

In some embodiments of the methods described herein, determining if at least one property of the gene product differs in the two cells comprise determining if the property of the gene differs by a qualitative measure. In other

10   embodiments, it comprises determining if it differs by a qualitative measure, such as by at least 10%, 20%, 40%, 50%, 75%, 100% or 200%.

In some embodiments, the property of a protein gene product comprises a physical property, such as its molecular weight, its isoelectric point, its amino acid

15   composition, its amino acid sequence, its dimerization state e.g. monomer, dimmer, tetramer, etc., or its association with other polypeptides to form a complex. Any techniques known in the art, such as mass spectrometry, SDS-PAGE, isoelectric focusing, peptide sequencing, gel filtration and immunoprecipitation may be used to measure these properties.

20

In some embodiments of the ongoing methods, an increase in a property of the gene product correlates with an increase in the number of biologically-active DNA-binding sites bound by the protein of interest, while in other embodiments it correlates with a decrease in the number of biologically-active DNA-binding sites

25   bound by the protein of interest. In other embodiments, a change in the property of the gene product correlates with a change in the biologically-active DNA-binding sites without altering the total number of sites.

In some preferred embodiments, the cells are tested in more than two

30   conditions, or under more than two candidate agents, such that a correlation may be extended to additional conditions or candidate agents.

V. Candidate agents

In some embodiments of the methods described herein, the candidate agent comprises a small molecule drug, an antisense nucleic acid, an antibody, a peptide, a ligand, a fatty acid, a hormone or a metabolite.

5

Exemplary compounds that may be used as candidate agents (e.g., a single compound, a combination of two or more compounds, a library of compounds) include nucleic acids, peptides, polypeptides, peptidomimetics, antibodies, antisense oligonucleotides, RNAi constructs (including siRNAs), ribozymes, chemical

10   compounds, and small organic molecules. Compounds may be screened individually, in combination, or as a library of compounds. Without being bound by theory, the invention contemplates that the modulation of cellular phenotypes may involve the activation or inhibition of particular genes and signaling pathways which modulate proliferation, survival, or differentiation along a particular lineage, thereby

15   modulating a cellular phenotype.

Candidate agents can be screened individually, in combination with one or more other compounds, or as a library of compounds. Compounds include nucleic acids, peptides, polypeptides, peptidomimetics, RNAi constructs, antisense

20   oligonucleotides, ribozymes, antibodies, and small molecules.

Antisense oligonucleotides are relatively short nucleic acids that are complementary (or antisense) to the coding strand (sense strand) of the mRNA encoding a particular protein. Although antisense oligonucleotides are typically

25   RNA based, they can also be DNA based. Additionally, antisense oligonucleotides are often modified to increase their stability.

Without being bound by theory, the binding of these relatively short oligonucleotides to the mRNA is believed to induce stretches of double stranded

30   RNA that trigger degradation of the messages by endogenous RNAses. Additionally, sometimes the oligonucleotides are specifically designed to bind near the promoter of the message, and under these circumstances, the antisense

oligonucleotides may additionally interfere with translation of the message. Regardless of the specific mechanism by which antisense oligonucleotides function, their administration to a cell or tissue allows the degradation of the mRNA encoding a specific protein. Accordingly, antisense oligonucleotides decrease the expression

5   and/or activity of a particular protein.

The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate

10   backbone, for example, to improve stability of the molecule, hybridization, *etc.* The oligonucleotide may include other appended groups such as peptides (*e.g.*, for targeting host cell receptors), or compounds facilitating transport across the cell membrane (see, *e.g.*, Letsinger *et al.*, 1989, Proc. Natl. Acad. Sci. U.S.A. 86:6553-6556; Lemaitre *et al.*, 1987, Proc. Natl. Acad. Sci. 84:648-652; PCT Publication No.

15   WO88/09810, published December 15, 1988) or the blood- brain barrier (see, *e.g.*, PCT Publication No. WO89/10134, published April 25, 1988), hybridization-triggered cleavage agents (See, *e.g.*, Krol *et al.*, 1988, BioTechniques 6:958- 976) or intercalating agents. (See, *e.g.*, Zon, 1988, Pharm. Res. 5:539-549). To this end, the oligonucleotide may be conjugated to another molecule.

20

The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5- bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5- (carboxyhydroxytriethyl) uracil, 5-carboxymethylaminomethyl-2-

25   thiouridine, 5- carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6- isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-

30   mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6- isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-

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methyluracil, uracil-5- oxyacetic acid methyl ester, uracil-5-oxyacetic acid (v), 5-
methyl-2-thiouracil, 3-(3-amino-3- N-2 -carboxypropyl) uracil, (acp3)w, and 2,6-
diaminopurine.

5   The antisense oligonucleotide may also comprise at least one modified sugar

moiety selected from the group including but not limited to arabinose, 2-
fluoroarabinose, xylulose, and hexose.   The antisense oligonucleotide can also
contain a neutral peptide-like backbone. Such molecules are termed peptide nucleic
acid (PNA)-oligomers and are described, e.g., in Perry-O'Keefe et al. (1996) Proc.
10   Natl. Acad. Sci. U.S.A. 93:14670 and in Eglom et al. (1993) Nature 365:566. One
advantage of PNA oligomers is their capability to bind to complementary DNA
essentially independently from the ionic strength of the medium due to the neutral
backbone of the DNA. In yet another embodiment, the antisense oligonucleotide
comprises at least one modified phosphate backbone selected from the group
15   consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a
phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl
phosphotriester, and a formacetal or analog thereof.

In yet a further embodiment, the antisense oligonucleotide is an -anomeric
20   oligonucleotide. An -anomeric oligonucleotide forms specific double-stranded
hybrids with complementary RNA in which, contrary to the usual -units, the strands
run parallel to each other (Gautier et al., 1987, Nucl. Acids Res. 15:6625-6641). The
oligonucleotide is a 2'-0-methylribonucleotide (Inoue et al., 1987, Nucl. Acids Res.
15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al., 1987, FEBS Lett.
25   215:327-330).

Oligonucleotides of the invention may be synthesized by standard methods
known in the art, e.g., by use of an automated DNA synthesizer (such as are
commercially available from Biosearch, Applied Biosystems, etc.). As examples,
30   phosphorothioate oligonucleotides may be synthesized by the method of Stein et al.
(1988, Nucl. Acids Res. 16:3209), methylphosphonate oligonucleotides can be
prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, Proc.

Natl. Acad. Sci. U.S.A. 85:7448-7451), *etc.*

       The selection of an appropriate oligonucleotide can be readily performed by one of skill in the art. Given the nucleic acid sequence encoding a particular protein,

5   one of skill in the art can design antisense oligonucleotides that bind to that protein, and test these oligonucleotides in an *in vitro* or *in vivo* system to confirm that they bind to and mediate the degradation of the mRNA encoding the particular protein. To design an antisense oligonucleotide that specifically binds to and mediates the degradation of a particular protein, it is important that the sequence recognized by

10   the oligonucleotide is unique or substantially unique to that particular protein. For example, sequences that are frequently repeated across protein may not be an ideal choice for the design of an oligonucleotide that specifically recognizes and degrades a particular message. One of skill in the art can design an oligonucleotide, and compare the sequence of that oligonucleotide to nucleic acid sequences that are

15   deposited in publicly available databases to confirm that the sequence is specific or substantially specific for a particular protein.

       In another example, it may be desirable to design an antisense oligonucleotide that binds to and mediates the degradation of more than one

20   message. In one example, the messages may encode related protein such as isoforms or functionally redundant protein. In such a case, one of skill in the art can align the nucleic acid sequences that encode these related proteins, and design an oligonucleotide that recognizes both messages.

25   A number of methods have been developed for delivering antisense DNA or RNA to cells; *e.g.*, antisense molecules can be injected directly into the tissue site, or modified antisense molecules, designed to target the desired cells (*e.g.*, antisense linked to peptides or antibodies that specifically bind receptors or antigens expressed on the target cell surface) can be administered systematically.

30

       However, it may be difficult to achieve intracellular concentrations of the antisense sufficient to suppress translation on endogenous mRNAs in certain

instances. Therefore another approach utilizes a recombinant DNA construct in which the antisense oligonucleotide is placed under the control of a strong pol III or pol II promoter. For example, a vector can be introduced *in vivo* such that it is taken up by a cell and directs the transcription of an antisense RNA. Such a vector can

5   remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the antisense RNA can be by

10   any promoter known in the art to act in mammalian, preferably human cells. Such promoters can be inducible or constitutive. Such promoters include but are not limited to: the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto *et al.*, 1980, Cell 22:787-797), the herpes thymidine kinase

15   promoter (Wagner *et al.*, 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster *et al*, 1982, Nature 296:39-42), *etc.* Any type of plasmid, cosmid, YAC or viral vector can be used to prepare the recombinant DNA construct that can be introduced directly into the tissue site. Alternatively, viral vectors can be used which selectively infect the

20   desired tissue, in which case administration may be accomplished by another route (*e.g.*, systematically).

RNAi constructs comprise double stranded RNA that can specifically block expression of a target gene. "RNA interference" or "RNAi" is a term initially

25   applied to a phenomenon observed in plants and worms where double-stranded RNA (dsRNA) blocks gene expression in a specific and post-transcriptional manner. Without being bound by theory, RNAi appears to involve mRNA degradation, however the biochemical mechanisms are currently an active area of research. Despite some mystery regarding the mechanism of action, RNAi provides a useful

30   method of inhibiting gene expression *in vitro* or *in vivo*.

As used herein, the term "dsRNA" refers to siRNA molecules, or other RNA

molecules including a double stranded feature and able to be processed to siRNA in cells, such as hairpin RNA moieties.

The term "loss-of-function," as it refers to genes inhibited by the subject
5   RNAi method, refers to a diminishment in the level of expression of a gene when compared to the level in the absence of RNAi constructs.

As used herein, the phrase "mediates RNAi" refers to (indicates) the ability to distinguish which RNAs are to be degraded by the RNAi process, *e.g.*,
10   degradation occurs in a sequence-specific manner rather than by a sequence-independent dsRNA response, *e.g.*, a PKR response.

As used herein, the term "RNAi construct" is a generic term used throughout the specification to include small interfering RNAs (siRNAs), hairpin RNAs, and
15   other RNA species which can be cleaved *in vivo* to form siRNAs. RNAi constructs herein also include expression vectors (also referred to as RNAi expression vectors) capable of giving rise to transcripts which form dsRNAs or hairpin RNAs in cells, and/or transcripts which can produce siRNAs *in vivo*.

20   "RNAi expression vector" (also referred to herein as a "dsRNA-encoding plasmid") refers to replicable nucleic acid constructs used to express (transcribe) RNA which produces siRNA moieties in the cell in which the construct is expressed. Such vectors include a transcriptional unit comprising an assembly of (1) genetic element(s) having a regulatory role in gene expression, for example, promoters,
25   operators, or enhancers, operatively linked to (2) a "coding" sequence which is transcribed to produce a double-stranded RNA (two RNA moieties that anneal in the cell to form an siRNA, or a single hairpin RNA which can be processed to an siRNA), and (3) appropriate transcription initiation and termination sequences. The choice of promoter and other regulatory elements generally varies according to the
30   intended host cell. In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer to circular double stranded DNA loops which, in their vector form are not bound to the chromosome.

In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

5

The RNAi constructs contain a nucleotide sequence that hybridizes under physiologic conditions of the cell to the nucleotide sequence of at least a portion of the mRNA transcript for the gene to be inhibited (*i.e.*, the "target" gene). The double-stranded RNA need only be sufficiently similar to natural RNA that it has the

10 ability to mediate RNAi. Thus, the invention has the advantage of being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism or evolutionary divergence. The number of tolerated nucleotide mismatches between the target sequence and the RNAi construct sequence is no more than 1 in 5 basepairs, or 1 in 10 basepairs, or 1 in 20 basepairs, or 1 in 50

15 basepairs. Mismatches in the center of the siRNA duplex are most critical and may essentially abolish cleavage of the target RNA. In contrast, nucleotides at the 3' end of the siRNA strand that is complementary to the target RNA do not significantly contribute to specificity of the target recognition.

20 Sequence identity may be optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, Sequence Analysis Primer, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default

25 parameters (*e.g.*, University of Wisconsin Genetic Computing Group). Greater than 90% sequence identity, or even 100% sequence identity, between the inhibitory RNA and the portion of the target gene is preferred. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript (*e.g.*, 400 mM NaCl, 40 mM

30 PIPES pH 6.4, 1 mM EDTA, 50 °C or 70 °C hybridization for 12-16 hours; followed by washing).

Production of RNAi constructs can be carried out by chemical synthetic methods or by recombinant nucleic acid techniques. Endogenous RNA polymerase of the treated cell may mediate transcription *in vivo*, or cloned RNA polymerase can be used for transcription *in vitro*. The RNAi constructs may include modifications to

5   either the phosphate-sugar backbone or the nucleoside, *e.g.*, to reduce susceptibility to cellular nucleases, improve bioavailability, improve formulation characteristics, and/or change other pharmacokinetic properties. For example, the phosphodiester linkages of natural RNA may be modified to include at least one of an nitrogen or sulfur heteroatom. Modifications in RNA structure may be tailored to allow specific

10   genetic inhibition while avoiding a general response to dsRNA. Likewise, bases may be modified to block the activity of adenosine deaminase. The RNAi construct may be produced enzymatically or by partial/total organic synthesis, any modified ribonucleotide can be introduced by *in vitro* enzymatic or organic synthesis.

15   Methods of chemically modifying RNA molecules can be adapted for modifying RNAi constructs (see, for example, Heidenreich *et al.* (1997) *Nucleic Acids Res*, 25:776-780; Wilson *et al.* (1994) *J Mol Recog* 7:89-98; Chen *et al.* (1995) *Nucleic Acids Res* 23:2661-2668; Hirschbein *et al.* (1997) *Antisense Nucleic Acid Drug Dev* 7:55-61). Merely to illustrate, the backbone of an RNAi construct

20   can be modified with phosphorothioates, phosphoramidate, phosphodithioates, chimeric methylphosphonate-phosphodiesters, peptide nucleic acids, 5-propynyl-pyrimidine containing oligomers or sugar modifications (*e.g.*, 2'-substituted ribonucleosides, a-configuration).

25   The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses (*e.g.*, at least 5, 10, 100, 500 or 1000 copies per cell) of double-stranded

30   material may yield more effective inhibition, while lower doses may also be useful for specific applications. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition.

- 53 -

In certain embodiments, the subject RNAi constructs are "small interfering RNAs" or "siRNAs." These nucleic acids are around 19-30 nucleotides in length, and even more preferably 21-23 nucleotides in length, e.g., corresponding in length
5   to the fragments generated by nuclease "dicing" of longer double-stranded RNAs. The siRNAs are understood to recruit nuclease complexes and guide the complexes to the target mRNA by pairing to the specific sequences. As a result, the target mRNA is degraded by the nucleases in the protein complex. In a particular embodiment, the 21-23 nucleotides siRNA molecules comprise a 3' hydroxyl group.
10

The siRNA molecules of the present invention can be obtained using a number of techniques known to those of skill in the art. For example, the siRNA can be chemically synthesized or recombinantly produced using methods known in the art. For example, short sense and antisense RNA oligomers can be synthesized and
15   annealed to form double-stranded RNA structures with 2-nucleotide overhangs at each end (Caplen, *et al.* (2001) *Proc Natl Acad Sci USA*, 98:9742-9747; Elbashir, *et al.* (2001) *EMBO J*, 20:6877-88). These double-stranded siRNA structures can then be directly introduced to cells, either by passive uptake or a delivery system of choice, such as described below.
20

In certain embodiments, the siRNA constructs can be generated by processing of longer double-stranded RNAs, for example, in the presence of the enzyme dicer. In one embodiment, the Drosophila *in vitro* system is used. In this embodiment, dsRNA is combined with a soluble extract derived from Drosophila
25   embryo, thereby producing a combination. The combination is maintained under conditions in which the dsRNA is processed to RNA molecules of about 21 to about 23 nucleotides.

The siRNA molecules can be purified using a number of techniques known
30   to those of skill in the art. For example, gel electrophoresis can be used to purify siRNAs. Alternatively, non-denaturing methods, such as non-denaturing column chromatography, can be used to purify the siRNA. In addition, chromatography

(e.g., size exclusion chromatography), glycerol gradient centrifugation, affinity purification with antibody can be used to purify siRNAs.

5   In certain preferred embodiments, at least one strand of the siRNA molecules has a 3' overhang from about 1 to about 6 nucleotides in length, though may be from 2 to 4 nucleotides in length. More preferably, the 3 ' overhangs are 1-3 nucleotides in length. In certain embodiments, one strand having a 3' overhang and the other strand being blunt-ended or also having an overhang. The length of the overhangs may be the same or different for each strand. In order to further enhance the stability of the

10   siRNA, the 3' overhangs can be stabilized against degradation. In one embodiment, the RNA is stabilized by including purine nucleotides, such as adenosine or guanosine nucleotides. Alternatively, substitution of pyrimidine nucleotides by modified analogues, e.g., substitution of uridine nucleotide 3' overhangs by 2'-deoxythyinidine is tolerated and does not affect the efficiency of RNAi. The absence

15   of a 2' hydroxyl significantly enhances the nuclease resistance of the overhang in tissue culture medium and may be beneficial in vivo.

In other embodiments, the RNAi construct is in the form of a long double-stranded RNA. In certain embodiments, the RNAi construct is at least 25, 50, 100,

20   200, 300 or 400 bases. In certain embodiments, the RNAi construct is 400-800 bases in length. The double-stranded RNAs are digested intracellularly, e.g., to produce siRNA sequences in the cell. However, use of long double-stranded RNAs in vivo is not always practical, presumably because of deleterious effects which may be caused by the sequence-independent dsRNA response. In such embodiments, the use

25   of local delivery systems and/or agents which reduce the effects of interferon or PKR are preferred.

In certain embodiments, the RNAi construct is in the form of a hairpin structure (named as hairpin RNA). The hairpin RNAs can be synthesized

30   exogenously or can be formed by transcribing from RNA polymerase III promoters in vivo. Examples of making and using such hairpin RNAs for gene silencing in mammalian cells are described in, for example, Paddison et al., Genes Dev, 2002,

16:948-58; McCaffrey *et al.*, *Nature*, 2002, 418:38-9; McManus *et al.*, *RNA*, 2002,

8:842-50; Yu *et al.*, *Proc Natl Acad Sci U S A*, 2002, 99:6047-52). Preferably, such

hairpin RNAs are engineered in cells or in an animal to ensure continuous and stable

suppression of a desired gene. It is known in the art that siRNAs can be produced by

5   processing a hairpin RNA in the cell.

In yet other embodiments, a plasmid is used to deliver the double-stranded

RNA, *e.g.*, as a transcriptional product. In such embodiments, the plasmid is

designed to include a "coding sequence" for each of the sense and antisense strands

10   of the RNAi construct. The coding sequences can be the same sequence, *e.g.*,

flanked by inverted promoters, or can be two separate sequences each under

transcriptional control of separate promoters. After the coding sequence is

transcribed, the complementary RNA transcripts base-pair to form the double-

stranded RNA.

15

PCT application WO01/77350 describes an exemplary vector for bi-

directional transcription of a transgene to yield both sense and antisense RNA

transcripts of the same transgene in a eukaryotic cell. Accordingly, in certain

embodiments, the present invention provides a recombinant vector having the

20   following unique characteristics: it comprises a viral replicon having two

overlapping transcription units arranged in an opposing orientation and flanking a

transgene for an RNAi construct of interest, wherein the two overlapping

transcription units yield both sense and antisense RNA transcripts from the same

transgene fragment in a host cell.

25

RNAi constructs can comprise either long stretches of double stranded RNA

identical or substantially identical to the target nucleic acid sequence or short

stretches of double stranded RNA identical to substantially identical to only a region

of the target nucleic acid sequence. Exemplary methods of making and delivering

30   either long or short RNAi constructs can be found, for example, in WO01/68836 and

WO01/75164.

Ribozyme molecules designed to catalytically cleave an mRNA transcript can also be used to prevent translation of mRNA (See, *e.g.*, PCT International Publication WO90/11364, published October 4, 1990; Sarver *et al.*, 1990, Science 247:1222-1225 and U.S. Patent No. 5,093,246). While ribozymes that cleave mRNA

5   at site-specific recognition sequences can be used to destroy particular mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead

10   ribozymes is well known in the art and is described more fully in Haseloff and Gerlach, 1988, Nature, 334:585-591.

The ribozymes of the present invention also include RNA endoribonucleases (hereinafter "Cech-type ribozymes") such as the one which occurs naturally in

15   Tetrahymena thermophila (known as the IVS, or L-19 IVS RNA) and which has been extensively described by Thomas Cech and collaborators (Zaug, *et al.*, 1984, Science, 224:574-578; Zaug and Cech, 1986, Science, 231:470-475; Zaug, *et al.*, 1986, Nature, 324:429-433; published International patent application No. WO88/04300 by University Patents Inc.; Been and Cech, 1986, Cell, 47:207-216).

20   The Cech-type ribozymes have an eight base pair active site that hybridizes to a target RNA sequence whereafter cleavage of the target RNA takes place. The invention encompasses those Cech-type ribozymes that target eight base-pair active site sequences.

25   As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (*e.g.*, for improved stability, targeting, *etc.*) and can be delivered to cells *in vitro* or *in vivo*. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the

30   ribozyme to destroy targeted messages and inhibit translation. Because ribozymes unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

WO 2005/088306

Antibodies can be used as inhibitors of the activity of a particular protein. Antibodies can have extraordinary affinity and specificity for particular epitopes. Antibodies that bind to a particular protein in such a way that the binding of the antibody to the epitope on the protein can interfere with the function of that protein. For example, an antibody may inhibit the function of the protein by sterically hindering the proper protein-protein interactions or occupying active sites. Alternatively the binding of the antibody to an epitope on the particular protein may alter the conformation of that protein such that it is no longer able to properly function.

Monoclonal or polyclonal antibodies can be made using standard protocols (See, for example, Antibodies: A Laboratory Manual ed. by Harlow and Lane (Cold Spring Harbor Press: 1988)). A mammal, such as a mouse, a hamster, a rat, a goat, or a rabbit can be immunized with an immunogenic form of the peptide. Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers or other techniques well known in the art.

Following immunization of an animal with an antigenic preparation of a polypeptide, antisera can be obtained and, if desired, polyclonal antibodies isolated from the serum. To produce monoclonal antibodies, antibody-producing cells (lymphocytes) can be harvested from an immunized animal and fused by standard somatic cell fusion procedures with immortalizing cells such as myeloma cells to yield hybridoma cells. Such techniques are well known in the art, and include, for example, the hybridoma technique (originally developed by Kohler and Milstein, (1975) Nature, 256: 495-497), the human B cell hybridoma technique (Kozbar et al., (1983) Immunology Today, 4: 72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., (1985) Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc. pp. 77-96). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with a particular polypeptide and monoclonal antibodies isolated from a culture comprising such hybridoma cells.

The term antibody as used herein is intended to include fragments thereof which are also specifically reactive with a particular polypeptide. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in

5   the same manner as described above for whole antibodies. For example, F(ab)$_2$ fragments can be generated by treating antibody with pepsin. The resulting F(ab)$_2$ fragment can be treated to reduce disulfide bridges to produce Fab fragments. The antibody of the present invention is further intended to include bispecific and chimeric molecules having affinity for a particular protein conferred by at least one

10   CDR region of the antibody.

Both monoclonal and polyclonal antibodies (Ab) directed against a particular polypeptides, and antibody fragments such as Fab, F(ab)$_2$, Fv and scFv can be used to block the action of a particular protein. Such antibodies can be used either in an

15   experimental context to further understand the role of a particular protein in a biological process, or in a therapeutic context.

Peptides, polypeptides, variants polypeptides, and peptide fragments can be candidate agents. Exemplary polypeptides comprise an amino acid sequence at least

20   60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99%, or 100% identical to a particular polypeptide. Exemplary fragments include fragments of at least 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 75, 100, 125, 150, 200, 250, or greater than 250 amino acid residues of the full length polypeptide. Peptides and polypeptides can either agonize or antagonize the function of a particular protein, and thereby modulate the phenotype

25   of a cell.

Small organic molecules can either agonize or antagonize the expression and/or activity of a particular protein, and thereby modulate the phenotype of a cell. By small organic molecule is meant a carbon contain molecule having a molecular

30   weight less than 2500 amu, more preferably less than 1500 amu, and even more preferably less than 750 amu. In the context of the present invention, such small organic molecules would be able to promote the differentiation of a cell to a

particular differentiated cell type.

Small molecules can be readily identified by screening libraries of organic molecules and/or chemical compounds to identify those compounds that have a desired function. Without being bound by theory, small organic molecules may influence a cellular phenotype in any of a number of ways. By way of example, small molecules may act at the cell surface to influence cell surface receptors. By way of further example, small molecules may act intracellularly to influence intracellular signaling along a particular signaling pathway. The methods of the present invention are unbiased and allow identification of small molecule compounds that modulate a cellular phenotype regardless of its mechanism of action.

In addition to compounds which are peptides or polypeptides, the invention contemplates nucleic acids comprising nucleotide sequences encoding peptides and polypeptides. The term nucleic acid as used herein is intended to include equivalents. The term equivalent is understood to include nucleotide sequences which are functionally equivalent to a particular nucleotide sequence. Equivalent nucleotide sequences will include sequences that differ by one or more nucleotide substitutions, additions or deletions, such as allelic variants, and variation due to degeneracy of the genetic code. Equivalent sequences may also include nucleotide sequences that hybridize under stringent conditions (*i.e.*, equivalent to about 20-27 °C below the melting temperature (T_m) of the DNA duplex formed in about 1M salt) to a given nucleotide sequence. Further examples of stringent hybridization conditions include a wash step of 0.2X SSC at 65 °C.

Nucleic acids having a sequence that differs from nucleotide sequences which encode a particular peptide or polypeptide candidate agent due to degeneracy in the genetic code are also within the scope of the invention. Such nucleic acids encode functionally equivalent peptides but differ in sequence from wildtype sequences known in the art due to degeneracy in the genetic code. For example, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid, or synonyms (for example, CAU and CAC each encode

histidine) may result in "silent" mutations which do not affect the amino acid sequence. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequences will also exist.

5   Biological conditions include any biological aspect of the shared fluid volume in which the cell populations are disposed. The biological aspects may include the presence, absence, concentration, activity, or type of cells, viruses, vesicles, organelles, biological extracts, and/or biological mixtures, among others. The assays described herein may screen a library of conditions to test the activity of

10   each library member on a set of cell populations. A library generally comprises a collection of two or more different members. These members may be chemical modulators (or candidate modulators) in the form of molecules, ligands, compounds, transfection materials, receptors, antibodies, and/or cells (phages, viruses, whole cells, tissues, and/or cell extracts), among others, related by any suitable or desired

15   common characteristic. This common characteristic may be "type." Thus, the library may comprise a collection of two or more compounds, two or more different cells, two or more different antibodies, two or more different nucleic acids, two or more different ligands, two or more different receptors, or two or more different phages or whole cell populations distinguished by expressing different proteins, among others.

20   This common characteristic also may be "function." Thus, the library may comprise a collection of two or more binding partners (*e.g.*, ligands and/or receptors), agonists, or antagonists, among others, independent of type.

          Library members may be produced and/or otherwise generated or collected

25   by any suitable mechanism, including chemical synthesis *in vitro*, enzymatic synthesis *in vitro*, and/or biosynthesis in a cell or organism. Chemically and/or enzymatically synthesized libraries may include libraries of compounds, such as synthetic oligonucleotides (DNA, RNA, peptide nucleic acids, and/or mixtures or modified derivatives thereof), small molecules (about 100 Da to 10 KDa), peptides,

30   carbohydrates, lipids, and/or so on. Such chemically and/or enzymatically synthesized libraries may be formed by directed synthesis of individual library members, combinatorial synthesis of sets of library members, and/or random

synthetic approaches. Library members produced by biosynthesis may include libraries of plasmids, complementary DNAs, genomic DNAs, RNAs, viruses, phages, cells, proteins, peptides, carbohydrates, lipids, extracellular matrices, cell lysates, cell mixtures, and/or materials secreted from cells, among others. Library

5   members may be contact arrays of cell populations singly or as groups/pools of two or more members.

EXEMPLIFICATION

The invention now being generally described, it will be more readily

10   understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention, as one skilled in the art would recognize from the teachings hereinabove and the following examples, that other DNA microarrays, transcriptional regulators, cell types, antibodies, ChIP conditions, or

15   data analysis methods, all without limitation, can be employed, without departing from the scope of the invention as claimed. The practice of the present invention will employ, where appropriate and unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, virology, recombinant DNA, and immunology, which are within the

20   skill of the art. Such techniques are described in the literature. See, for example, Molecular Cloning: A Laboratory Manual, 3rd Ed., ed. by Sambrook and Russell (Cold Spring Harbor Laboratory Press: 2001); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Using Antibodies, Second Edition by Harlow and Lane, Cold Spring Harbor Press, New York, 1999; Current Protocols in Cell

25   Biology, ed. by Bonifacino, Dasso, Lippincott-Schwartz, Harford, and Yamada, John Wiley and Sons, Inc., New York, 1999; and PCR Protocols, ed. by Bartlett *et al.*, Humana Press, 2003.

Various publications, patents, and patent publications are cited throughout

30   this application, the contents of which are incorporated herein by reference in their entirety.

Experimental Procedures:

The following procedures were followed for the experimental examples.

Genetic Reagents

5   The 203 transcriptional regulators were identified by searching the YPD and

MIPS databases for known and predicted transcription factors and nucleic acid

binding proteins (Mewes, H. W., Albermann, K., Heumann, K., Liebl, S. & Pfeiffer,

F. MIPS: a database for protein sequences, homology data and yeast genome

information. *Nucleic Acids Res* 25, 28-30 (1997); Hodges, P. E., McKee, A. H.,

10   Davis, B. P., Payne, W. E. & Garrels, J. I. The Yeast Proteome Database (YPD): a

model for the organization and presentation of genome-wide functional data. *Nucleic

Acids Res* 27, 69-73 (1999); Costanzo, M. C. *et al.* YPD, PombePD and WormPD:

model organism volumes of the BioKnowledge library, an integrated resource for

protein information. *Nucleic Acids Res* 29, 75-9 (2001)). Yeast strains were created

15   for each of the 203 regulators in which a repeated Myc epitope coding sequence was

integrated into the endogenous gene encoding the regulator. PCR constructs

containing the Myc epitope coding sequence and a selectable marker flanked by

regions of homology to either the 5' or 3' end of the targeted gene were transformed

into the W303 yeast strain Z1256. Genomic integration and expression of the

20   epitope-tagged protein were confirmed by PCR and Western blotting, respectively.

Growth conditions

Regulators were selected for profiling in a specific environment if they were

essential for growth in that environment or if there was other evidence implicating

25   them in regulation of gene expression in that environment.

A brief description of the environmental conditions used follows:

Rich media. Cells were grown in YPD (1% yeast extract/2% peptone/2% glucose) to

30   an OD600 of ~0.8.

Highly hyperoxic. Cells were grown in YPD to an OD600 of ~0.5 followed by

treatment with hydrogen peroxide (4 mM final) for 30 minutes.

Moderately hyperoxic. Cells were grown in YPD to an OD600 of ~0.5 followed by treatment with hydrogen peroxide (0.4 mM final) for 20 minutes.

5

Amino acid starvation. Cells were grown to an OD600 of ~0.6 in synthetic complete medium followed by treatment with the inhibitor of amino acid biosynthesis sulfometuron methyl (0.2 μg/ml final) for two hours.

10   *Nutrient deprived.* Cells were grown in YPD to an OD600 of ~0.8 followed by treatment with rapamycin (100 nM final) for 20 minutes.

Filamentation inducing. Cells were grown in YPD containing 1% butanol for either 90 minutes or 14 hours (corresponding to an OD600 of ~0.8).

15

Mating inducing. Cells were grown in YPD to an OD600 of ~0.8 followed by treatment with the alpha factor pheromone (5 μg/ml) for 30 minutes.

Elevated temperature. Cells were grown in YPD at 30°C to an OD600 of ~0.5

20   followed by a temperature shift to 37°C for 45 minutes.

Galactose medium. Cells were grown in YEP medium supplemented with galactose (2%) to an OD600 of ~0.8.

25   *Raffinose medium.* Cells were grown in YEP medium supplemented with raffinose (2%) to an OD600 of ~0.8.

Acidic medium. Cells were grown in YPD to an OD600 of ~0.5 followed by treatment for 30 minutes with succinic acid (0.05 M final) to reach a pH of 4.0.

30

Phosphate deprived medium. Cells were grown in synthetic complete medium lacking phosphate to a final OD600 of ~0.8.

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Vitamin deprived medium. Cells were grown in synthetic complete medium lacking thiamine to a final OD600 of ~0.8.

5   Strain Information

For each of the 203 regulators, strains were generated in which a repeated Myc epitope coding sequence was integrated into the endogenous gene encoding the regulator. Polymerase chain reaction (PCR) constructs containing the Myc epitope coding sequence and a selectable marker flanked by regions of homology to either
10   the 5' or 3' end of the targeted gene were transformed into the W303 yeast strain Z1256. Genomic integration and expression of the epitope-tagged protein were confirmed by PCR and Western blotting, respectively.

Genome-wide Location Analysis

15   Genome-wide location analysis was performed as previously described (Orlando, V. Mapping chromosomal proteins *in vivo* by formaldehyde-crosslinked-chromatin immunoprecipitation. *Trends Biochem Sci* 25, 99-104 (2000); Tessier, D. *et al. A DNA Microarrays Fabrication Strategy for Research Laboratories.* (eds. Rehm, H. & Reed, G.) (Wiley-VCH, Weinheim, Germany, 2002)). Bound proteins
20   were formaldehyde-crosslinked to DNA *in vivo*, followed by cell lysis and sonication to shear DNA. Crosslinked material was immunoprecipitated with an anti-myc antibody, followed by reversal of the crosslinks to separate DNA from protein. Immunoprecipitated DNA and DNA from an unenriched sample were amplified and differentially fluorescently labelled by ligation-mediated PCR. These
25   samples were hybridized to a microarray consisting of spotted PCR products representing the intergenic regions of the S. cerevisiae genome. Relative intensities of spots were used as the basis for an error model that assigns a probability score (P value) to binding interactions. All microarray data is available from ArrayExpress (accession number: E-WMIT-10) as well as from the authors' web site.

30

Growth Environments

Applicants profiled all 203 regulators in rich medium. In addition,

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Applicants profiled 84 regulators in at least one other environmental condition. The list of regulators is given in Table 1.

Microarray Design

5   Using the Yeast Intergenic Region Primer set (Research Genetics) Applicants PCR amplified and printed approximately 6000 DNA fragments, representing essentially all of the known intergenic regions in the yeast genome (Tessier, D. *et al. A DNA Microarrays Fabrication Strategy for Research Laboratories.* (eds. Rehm, H. & Reed, G.) (Wiley-VCH, Weinheim, Germany,

10   2002)). The average size of the spotted PCR products was 480 bp, and the sizes ranged from 60 bp to 1500 bp.

Raw Data Analysis

         The microarrays were scanned using an Axon200B scanner, and the images

15   were analyzed with Genepix 5.0. Columns corresponding to the background subtracted intensities and standard deviation of the background were extracted for further analysis. The intensities for the two channels, representing the immunoprecipitated (test) and unenriched (control) samples, were normalized by using the median of each channel to calculate a normalization factor, normalizing all

20   datasets to a single median intensity. The log ratio of the intensity in the test channel to the control channel was calculated. To account for biases in the immunoprecipitation reaction, these log ratios were normalized for each spot by subtracting the average log ratio of each spot across all arrays. The intensities in the test channel were then adjusted to yield this normalized ratio. Finally, an error

25   model (Hughes *et al.*(2000) *Cell* 102, 109-26) was used to calculate significance of enrichment on each chip and to combine data for replicates to obtain a final average ratio and significance of enrichment for each intergenic region. Each intergenic region was assigned to the genes it is most likely to regulate, as described on the author's website.

30

         Applicants have included new refinements in our analysis relative to that used in Lee *et al. Science* 298, 799-804. (2002). Notably, Applicants have excluded

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artefactual spots from analysis, selected more reliable probes for normalization and assigned quality metrics to individual arrays to identify low quality experiments.

Error Estimates

5   Applicants previously estimated a false positive rate of 6-10% for genome-wide binding data that meets a $P \leq 0.001$ threshold. The present study is focused on DNA regions that are both bound ($P \leq 0.001$) and contain a conserved match to a binding site specificity. Of 47 sites that were used by Lee *et al.* *Science* 298, 799-804. (2002), to determine the error rate and that met our criteria for binding sites, 45

10   were confirmed by independent gene-specific ChIP experiments. Thus, the frequency of false positives in this dataset is likely to be approximately 4%.

The false negative rate is more difficult to estimate, but it is likely to be approximately 24% in the present genome location dataset. This estimate was

15   derived by determining the number of binding interactions reported in the literature for cell cycle regulators that were not identified in the genome-wide location data at $P \leq 0.001$ and associated with conserved binding sites (12/50). Applicants selected the cell cycle literature for analysis because of the extensive study of this group of regulators and their targets.

20

Regulator Binding Specificity

         The putative specificities of regulators were identified by applying a suite of motif discovery programs to the intergenic sequences identified by the binding data. The resulting specificity predictions were filtered for significance using uniform

25   metrics and then clustered to yield representative motifs (Figure 6). Applicants used six methods to identify the specific sequences bound by regulators: AlignACE11, MEME13, MDscan12, the method of Kellis *et al.* and two additional new methods that incorporate conservation data: MEME_c and CONVERGE. MEME_c uses the existing MEME program without change, but applies it to a modified set of

30   sequences in which bases that are not conserved in the *sensu stricto* Saccharomyces species were replaced with the letter "N". CONVERGE is a novel expectation-maximization (EM)-based algorithm for discovering specificities using sequence

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information from multiple genomes. Rather than searching for sites that are identical across the sensu stricto species, as is the case for MEME_c, CONVERGE searches for loci where all aligned sequences are consistent with the same specificity model.

5   Each of the programs Applicants used attempts to measure the significance of its results with one or more statistical scores. However, Applicants observed that these programs report results with high scores even when applied to random selections of intergenic regions. To distinguish the true motifs, Applicants chose a set of statistical measures as described in the experimental procedures, and

10   Applicants converted these scores into the empirical probability that a motif with a similar score could be found by the same program in randomly selected sequences. To estimate these P values, Applicants ran each program 50 times on randomly selected sets of sequences of various sizes. Applicants accepted only those motifs that were judged to be significant by these scores (P ≤0.001).

15

Significant motifs from all programs were pooled together and clustered using a k-medoids algorithm. Aligned motifs within each cluster were averaged together to produce consensus motifs and filtered according to their conservation. This procedure typically produced several distinct consensus motifs for each

20   regulator. To choose a single specificity for each regulator, Applicants compared the results with information in the TRANSFAC27, YPD28, and SCPD29 databases. When no prior information was available, Applicants chose the specificity with the most significant statistical score.

25   Motif Discovery Overview
Binding motifs were identified in a five-step process described in detail below and summarized in Figure 6. First, motifs were discovered by applying a suite of motif discovery programs to the intergenic sequences identified by the binding data. The resulting specificity predictions were filtered for significance

30   using uniform metrics and then clustered to yield representative motifs. Conservation-based metrics were used to identify the highest-confidence subset of these motifs. For cases in which multiple significant binding motifs were found for

a factor, Applicants used statistical scores or information from the Transfac (Matys, V. *et al*. TRANSFAC: transcriptional regulation, from patterns to profiles. *Nucleic Acids Res* 31, 374-8 (2003)), YPD (Csank, C. *et al*. Three yeast proteome databases: YPD, PombePD, and CalPD (MycoPathPD). *Methods Enzymol* 350, 347-73 (2002)),

5 and SCPD (Zhu, J. & Zhang, M. Q. SCPD: a promoter database of the yeast Saccharomyces cerevisiae. *Bioinformatics* 15, 607-11 (1999)) databases to choose a single motif for each regulator. Sequence input files, intermediate motif discovery output, and matrix representations of the finalized motifs are available on the authors' website.

10

Step 1: Initial Motif Discovery

Motif Discovery Programs have different strengths with respect to finding specificities. To gain as comprehensive an analysis as possible, Applicants applied five different motif-finding programs to the binding data: AlignACE (Roth, F. P.,

15 Hughes, J. D., Estep, P. W. & Church, G. M. Finding DNA regulatory motifs within unaligned noncoding sequences clustered by whole-genome mRNA quantitation. *Nat Biotechnol* 16, 939-45 (1998)), MEME (Bailey, T. L. & Elkan, C. The value of prior knowledge in discovering motifs with MEME. *Proc Int Conf Intell Syst Mol Biol* 3, 21-9 (1995)), MDscan (Liu, X. S., Brutlag, D. L. & Liu, J. S. An algorithm

20 for finding protein-DNA-binding sites with applications to chromatin-immunoprecipitation microarray experiments. *Nat Biotechnol* 20, 835-9 (2002)), the conservation-based method described in Kellis *et al*. (Kellis, M., Patterson, N., Endrizzi, M., Birren, B. & Lander, E. S. Sequencing and comparison of yeast species to identify genes and regulatory elements. *Nature* 423, 241-54 (2003)) , and

25 a new conservation-based method called CONVERGE (described below). The MEME program was also used to analyze a modified input that incorporated conservation information (see "Probe Sequences").

To make the search more thorough, each of these programs was run multiple

30 times with different parameters. AlignACE was run using the default settings ten times with different random number seeds, in order to increase the motif space it sampled. The results from the AlignACE runs were grouped together for analysis.

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MEME was run using the supplied 5th-order Markov background model, the "ZOOPS" motif model, and the "-minsites 20 -DNA -revcomp" options. MEME runs were repeated using motif width ranges of 7 to 11 and 12 to 18. To run MDscan, sequences were ranked according the P-value of binding, and the program

5   was run with the "-s 30 -r 5 -t 10" options. To compensate for the fact that MDscan searches only for motifs of fixed width, the program was run repeatedly, once with each width in the range 8 to 15 bases. The method of Kellis *et al.* was applied to the data as described (Kellis, M., Patterson, N., Endrizzi, M., Birren, B. & Lander, E. S. Sequencing and comparison of yeast species to identify genes and regulatory

10   elements. *Nature* **423**, 241-54 (2003)). CONVERGE was run twice using motif widths of 8 and 15.

MEME_c

Applicants tested whether Applicants could improve the performance of

15   AlignACE, MEME and MDscan by modifying the input sequences to convey the conservation of each base in the *sensu stricto Saccharomyces* species. Using ClustalW (Thompson, J. D., Higgins, D. G. & Gibson, T. J. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice.

20   *Nucleic Acids Res* **22**, 4673-80 (1994)) alignments for the *sensu stricto* species (Kellis, M., Patterson, N., Endrizzi, M., Birren, B. & Lander, E. S. Sequencing and comparison of yeast species to identify genes and regulatory elements. *Nature* **423**, 241-54 (2003)), Applicants replaced a base in the *Saccharomyces* genome with the letter "N" if it was not conserved in 2/3 or 3/4 of the other genomes. Of the

25   programs tested, only MEME was able to use the modified sequences.

CONVERGE

Applicants designed CONVERGE to identify motifs that are both over-represented in a set of input sequences and conserved across multiple genomes.

30   CONVERGE input sequences consists of an ungapped DNA sequence corresponding to the primary genome, as well as one or more optional aligned sequences, which may contain gaps. The algorithm is based on the ZOOPS model

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of MEME and uses a 5th-order Markov background model. However, whereas MEME searches for matches to a motif model across a set of input sequences, CONVERGE searches across the multiple-sequence alignments for each sequence. Specifically, CONVERGE treats the probability of a motif occurring at a site in the

5   alignment as the product of the probabilities of the motif occurring at the same site in each of the aligned sequences. Thus, CONVERGE defines a site as conserved in a flexible manner that depends on the motif being discovered. Full details will be presented elsewhere.

10   *Probe Sequences*

Motif discovery programs were applied to the sequences of probes bound with a P-value ≤ 0.001. Applicants found that some intergenic regions were highly homologous over their entire length, and consequently skew the results of motif discovery since all subsequences are overrepresented. To remove this bias, BLAST

15   (Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. Basic local alignment search tool. *J Mol Biol* **215**, 403-10 (1990)) was used to identify pairs of probes with high sequence similarity over 50% of their lengths. For each pair, the shorter intergenic region was omitted from motif discovery computations. This process removed up to nine regions for some experiments, but less than one on

20   average.

To determine the sequences present on the microarrays, Applicants computed the expected products of the PCR used to construct the arrays. Research Genetics primer sequences were obtained from

25   http://www.resgen.com/products/YeIRP.php3 and the March 2002 revision of the yeast genome was obtained from SGD (Dwight, S. S. *et al. Nucleic Acids Res* **30**, 69-72 (2002)). Probes that were predicted to amplify more than two different genomic sequences were omitted from the calculations. Twenty five probe sequences neighboring repetitive, non-transcribed features (*e.g.* telomeric repeats, X

30   elements and Y' elements) were also omitted.

PSSM Representation

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Motifs from all programs were converted to a standard position-specific scoring matrix (PSSM) for subsequent analysis. AlignACE and MDscan produce alignments of binding sites, and these were first converted into matrices representing the frequency of each base (A, C, G, T) at each position of the alignments. The

5   method of Kellis *et al.* represents motifs as text strings containing ambiguity codes, which were also converted to matrices of frequencies. (For example, if a motif contained the letter "S" at a particular position, a value of 0.5 would be assigned to both "C" and "G.") The matrices of base frequencies were converted to probabilities and then were adjusted with 0.001 pseudo-counts in proportion to the

10   0th-order background probabilities (3.1×10^{-4} pseudocounts for A and T, 1.9×10^{-4} pseudocounts for G and C). Log-likelihood scores were computed by dividing the estimated probabilities by the background probability for each letter and computing the base-2 logarithm. CONVERGE and MEME both provide probability matrices, which were used directly.

15

Step 2: Motif Scoring and Significance Testing

Applicants tested the significance of each motif by comparing how often it was found in the bound and unbound probes. To encapsulate different approaches to measuring motif over-representation, Applicants employed three different metrics:

20   Enrichment, ROC AUC, and for motifs discovered by the method described in Kellis *et al.*, the "CC4" score. The enrichment score is a direct measure of the occurrence of a motif among bound probes compared to all possible gene targets, but does not distinguish between the number of motifs occurrences within each intergenic region. The ROC AUC metric is more sensitive to cases in which the

25   number of motif occurrences is a distinguishing factor. Finally, the CC4 metric provides a way to account for the importance of the conservation of the motif among bound probes. These scores were compared to significance thresholds obtained from calculations on randomized selections of intergenic regions as described below in "Significance Thresholds"

30

Enrichment score

To obtain the enrichment score, the hypergeometric distribution was used to

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compare the frequency of the motif in the bound probes to that which would be
expected if the intergenic regions were selected at random from the genome. A
sequence was considered to contain a motif if it contained at least one or more sites
scoring at least 70% of the maximum possible score of the matrix. A P-value for the
5   enrichment was computed according to the formula:

$$p = \sum_{i=b}^{\min(B,g)} \frac{\binom{B}{i}\binom{G-B}{g-i}}{\binom{G}{g}}$$

(5)

where B is the number of bound intergenic regions and G is the total number of
intergenic regions represented on the microarray (or the genome). The quantities b
and g represent the number of intergenic regions of B and G matching the motif. The
10   quantity $-log_{10}(p)$ is referred to as the enrichment score.

ROC AUC (Receiver Operating Characteristic Area Under Curve)

The ROC AUC refers to the area under a receiver operating characteristic
curve which is assembled by ranking the sets of bound and unbound probes
15   according to the number of motif matches they contain, and plotting the fractional
rankings against each other. Applicants used the method and code described by
Clarke and Granek (Clarke, N. D. & Granek, J. A. Rank order metrics for
quantifying the association of sequence features with gene regulation.
Bioinformatics 19, 212-8 (2003)).

20

Conservation CC4

Motifs discovered using the method of Kellis *et al.* (Kellis, M., Patterson, N.,
Endrizzi, M., Birren, B. & Lander, E. S. Sequencing and comparison of yeast
species to identify genes and regulatory elements. *Nature* 423, 241-54 (2003)) were
25   judged according to the CC4 metric, in which the occurrence of a conserved motif
among the bound probes is compared to the expected ratio observed among all 3-
gap-3 motifs in among the same set of bound probes. The binomial probability of
the observed ratio was computed, and is reported in terms of the equivalent z-score.

Significance Thresholds

Applicants observed that motif discovery programs produce motifs with high over-representation metrics (such as "Enrichment" and "ROC AUC") even when applied to random selections of intergenic regions. To identify the true motifs,

5   Applicants converted the scores from each metric into the empirical probability that a motif with a similar score could be found by the same program in randomly selected sequences. Applicants accepted only those motifs with a P-value ≤ 0.001. Applicants selected this stringent threshold to minimize false positives, and because Applicants observed empirically that it identified the correct motifs for many

10   regulators with known specificity. To estimate these thresholds, Applicants ran each program 50 times on randomly selected sequences on sets of 10, 20, 30, 40, 50, 60, 70, 80, 100, 120, 140, and 160 probes.

The observed scores from these random runs were parameterized by a

15   normal distribution. The critical values equivalent to a P-value of 0.001 are provided in Table 8 for each program and each metric. If the empirical distribution was not normal (by the Shapiro-Wilk test), the corresponding metric was not used to evaluate motifs generated by the relevant program for regulators with a similar number of bound probes.

20

For a particular experiment, Applicants employed the threshold derived from the randomization set that had the size closest to the number of bound probe sequences. For example, suppose a motif found by performing ten runs of AlignACE on 32 intergenic sequences had an enrichment score of 25. The relevant

25   score distribution has been obtained by performing ten runs of AlignACE on each of 50 randomly selected sets of 30 intergenic sequences. The resulting distribution of enrichment scores has a mean of 14.1 and standard deviation of 2.1, and the enrichment that corresponds to significance of $P \leq 0.001$ is thus 20.43. Since the score of the candidate motif is higher, it is considered significant.

30

Step 3: Motif Clustering and Averaging

K-medoids Clustering

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The set of significant motifs for each experiment was then clustered via k-medoids clustering (Hastie *et al. The elements of Statistical Learning; Data mining, inference and prediction* (Springer-Verlag, New York, 2001)) using the distance metric described below. The k-medoids algorithm was performed 500 times to find

5   a clustering with a minimal sum of inter-cluster distances. To find the optimal number of clusters, this process was first performed with 10 clusters, and then repeated with incrementally fewer clusters until all average distances between members of a cluster and medoids of other clusters were sufficiently large (greater or equal to 0.18).

10

Inter-Motif Distance

Applicants constructed a distance metric to aid in the comparison of motifs. The distance D between two aligned motifs "a" and "b" is defined as,

$$D(a,b) = \frac{1}{w}\sum_{i=1}^{w}\frac{1}{\sqrt{2}}\sum_{L \in \{ACGT\}}(a_{i,L} - b_{i,L})^2$$

15   (1)

where w is the motif width, and $a_{i,L}$ and $b_{i,L}$ are the estimated probabilities of observing base L at position i of motifs a and b, respectively. The normalizations by

w and $\sqrt{2}$ facilitate the interpretation as a fractional distance. For example, a distance of 0.20 indicates that the two motifs differ by about 20%.

20

In practice, the optimal alignment of motifs is not known. Applicants therefore use the minimum distance between motifs among all alignments in which the motifs overlap by at least seven bases, or when the motifs are shorter, by 2 bases fewer than the shortest motif length. Alignments to the reverse complements of the

25   motifs are included.

Motif Averaging

A single motif representing each cluster was computed by averaging the probabilities at each matrix position of the aligned motifs comprising the cluster.

30   Low-information positions on the flanks of the averaged motifs were removed.

Step 4: Conservation Testing for Averaged Motifs

Applicants tested the conservation of averaged motifs, and focused subsequent analysis on the motifs that met two conservation criteria: First, Applicants required that the frequency of conserved instances of the motif compared

5   to all instances of the motif be at least as high within bound intergenic regions as among all intergenic regions. Second, Applicants required that discovered motifs have at least three conserved instances that are bound.

Applicants considered a sequence a match to a motif if it had a score of at

10   least 60% of the motif maximum. Applicants defined a "conserved instance" to mean that the aligned sequence of at least two other *sensu stricto* species also matched the motif. In cases where fewer than two aligned sequences were available, a site was treated as "not conserved."

15   Step 5: Assignment a Single Motif to Each Regulator

Often, the motif discovery process produced several significant, distinct averaged motifs (3 on average.). These motifs could represent the desired binding specificity of the protein, or they might arise from the specificity of binding partners or have other biological significance. To identify those motifs representing the

20   binding specificity of the profiled transcription factor, Applicants compared the specificities to binding data in the Transfac (Matys, V. *et al.* TRANSFAC: transcriptional regulation, from patterns to profiles. *Nucleic Acids Res* 31, 374-8 (2003)), YPD (Csank, C. *et al.* Three yeast proteome databases: YPD, PombePD, and CalPD (MycoPathPD). *Methods Enzymol* 350, 347-73 (2002)), and SCPD (Zhu,

25   J. & Zhang, M. Q. SCPD: a promoter database of the yeast Saccharomyces cerevisiae. *Bioinformatics* 15, 607-11 (1999)) databases, when available, using the same inter-motif distance metric used for clustering (see above.) There were 21 regulators for which no such data were available. In these cases Applicants chose the motif with the best enrichment score.

30

Specificity data from these databases is sometimes available in the forms of raw sequences, ambiguity codes, and matrices. For regulators without matrices,

Applicants assembled a single consensus sequence to represent the body of
experimentally determined specificity information and converted it to a PSSM as
described above. Since there is no way to independently assess the quality of the
motifs assembled from the databases, Applicants used a permissive threshold to
5   detect similarity between the discovered motifs and the database motifs. Motifs
scoring below 0.24 were accepted as matches, while motifs with scores less than
0.35 were examined manually. The scores for the motifs that were used in the
Regulatory Code Map are provided in Table 2.

10   Motifs Derived from the Literature

Applicants used a motif derived from the databases for the remaining
regulators for which either: (1) Too few intergenic regions (<10) were bound for
effective motif discovery, (2) discovered motifs similar to the literature were
eliminated by the conservation in Step 4, or (3) none of the discovered motifs
15   matched the literature in Step 5. These motifs were only included if they had at least
one conserved instance that was bound. The resulting compendium of 102 motifs
(Table 3) was used in all subsequent analysis.

Regulatory Code Map
20   Binding motifs for 102 regulators (Table 3) were fused with location analysis
data and conservation data to produce a map of active binding sites in intergenic
regions. The entire map is available at
http://web.wi.mit.edu/fraenkel/regulatory_map/. The map was constructed by
finding all conserved occurrences of each motif within intergenic regions bound by
25   the corresponding factor.

Applicants used a binding P-value threshold of $P \leq 0.001$ and the definition
of conservation as described in the "Conservation Test" section above. Variants of
the map constructed with different binding and conservation thresholds are also
30   available online.

Distributions of distances from the start codon (ATG) of open reading frames

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to binding sites in the adjacent upstream region were derived from the above data. These were compared to a distribution calculated on ten thousand "randomized" genomes in which the binding sites in each intergenic region were redistributed randomly and independently between the adjacent genes. The region from −100 to −

5   500 (grey area in Figure 2C) contains many more binding sites than expected.

Promoter Classification

Promoters were classified based on the aggregate binding data from all experiments. A promoter was defined as having multiple regulator architecture if

10   more than one regulator bound in the aggregate data, regardless of the number of regulators that bound in any particular condition. Similarly, a promoter was assigned to the single regulator architecture if it was bound by exactly one regulator in the aggregate data.

15   Regulators that had a tendency to use the repetitive motif architecture were identified by chi-square analysis. For each regulator, Applicants calculated the number of promoters containing a single site and the number containing multiple sites. These values were then compared to the expected values based on the average for all factors.

20

Co-occurring regulatory motifs were determined based on P values representing the probability, based on the hypergeometric distribution, of finding the observed number of intergenic regions (or more) bound by both regulators under the null hypothesis that binding for the two regulators is independent.

25

Regulator Behavior Classification

The binding of each regulator was compared in pair-wise fashion for every environmental condition in which that regulator was studied. Only regions bound at $P \leq 0.001$ and containing conserved matches to the corresponding motif were

30   included in this analysis. Some regulators fall into multiple categories depending on exactly which conditions are compared. For the "condition invariant" category the ratio of the overlap of bound probes for a regulator was greater than 0.66, and the

ratio of the number of bound probes was between 0.66 and 1.5. For the "condition enabled" category the regulator bound to no probes in one environment. For the "condition expanded" category the ratio of the overlap of bound probes for a regulator was greater than 0.66, and the ratio of the number of bound probes was

5   less than 0.66 or greater than 1.5. For the "condition altered" category the regulator bound at least one probe in both environments and the ratio of the overlap of bound probes was less than 0.66.

Experimental Confirmation of Predicted Specificity

10   Applicants compared the discovered motifs to those in the literature using an automated method, and selected the regulator for which the discrepancy was the greatest, Cin5 (Table 2). The discovered motif, TTAcrTAA, contains a one base insertion compared to the previously reported site (Fernandes, L., Rodrigues-Pousada, C. & Struhl, K. Yap, a novel family of eight bZIP proteins in

15   Saccharomyces cerevisiae with distinct biological functions. *Mol Cell Biol* 17, 6982-93 (1997)), TTACTAA. The previously known site is poorly enriched in the probes bound by Cin5 ($P \leq 0.02$), while the discovered motif is very strongly enriched ($P \leq 10^{-38.4}$).

20   Applicants used a gel-shift assay to test whether the specificity for Cin5 that Applicants inferred from our *in vivo* data also represented the *in vitro* properties for this regulator (Figure 7). The DNA–binding domain of Cin5 was cloned into a derivative of the pET-32 vector (Novagen) fused to thioredoxin and a poly-histidine peptide, expressed in *E. coli*, and purified by affinity chromatography. Protein was

25   incubated with a Cy5-labeled oligonucleotide containing the sequence gcgacaTTACCTAAgggc and challenged with unlabeled competitor containing either the same sequence or the previously published binding site (gcgacaTTACTAAagggc) (Fernandes*et al. Mol Cell Biol* 17, 6982-93 (1997)). The reactions were analyzed on 10% acrylamide gels run in 0.5x TBE. Similar results

30   were obtained for a probe containing the core sequence of TTACGTAA.

Regulatory Code

Potential binding sites were included in the map of the regulatory code if they satisfied two criteria. First, a locus had to match the specificity model for a regulator in the Saccharomyces cerevisiae genome and at least two other sensu stricto cerevisiae genomes with a score ≥60% of the maximum possible. Second,

5   the locus had to lie in an intergenic region that also contained a probe bound by the corresponding regulator in any condition (P ≤0.001). All analyses of promoter architecture and environment-specific binding were based on this map, and is described further below. More detailed information concerning all the methods described in the example below can be found in at

10   http://web.wi.mit.edu/young/regulatory_code.

Example 1: Identification of Biologically-Active DNA-binding Sites

Applicants used genome-wide location analysis (Iyer *et al. Nature* 409, 533-8. (2001), Ren *et al. Science* 290, 2306-9. (2000), Lee *et al. Science* 298, 799-804.

15   (2002), Lieb *et al. Nat Genet* 28, 327-34 (2001)) to determine the genomic occupancy of 203 DNA-binding transcriptional regulators in rich media conditions and, for 84 of these regulators, in at least one of twelve other environmental conditions (Table 1, Figure 5, http://web.wi.mit.edu/young/regulatory_code). These 203 proteins are likely to include nearly all of the DNA-binding transcriptional

20   regulators encoded in the yeast genome. Regulators were selected for profiling in an additional environment if they were essential for growth in that environment or if there was other evidence implicating them in regulation of gene expression in that environment. The genome-wide location data identified 11,000 unique interactions between regulators and promoter regions at high confidence (P ≤0.001).

25

To identify the cis-regulatory sequences that likely serve as recognition sites for transcriptional regulators, Applicants merged information from genome-wide location data, phylogenetically conserved sequences, and prior knowledge (Figure 1A). Applicants used six motif discovery methods11-13 to discover 68,279 DNA

30   sequence motifs for the 147 regulators that bound more than ten probes (see experimental procedures; Figure 6). From these motifs Applicants derived the most likely specificity for each regulator through clustering and stringent statistical tests.

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This motif discovery process identified highly significant ($P \leq 0.001$) motifs for each of 116 regulators. Applicants determined a single high-confidence motif for 65 of these regulators using additional criteria including the requirement for conservation across three of four related yeast species. Examples of novel and "re-

5 discovered" motifs are depicted in Figure 1B, and comparisons of the discovered motifs to those described previously are shown in Table 2. The discovered motifs provide significantly more information than was previously available; for 21 of the regulators there was no prior specificity information in the literature, and detailed probability matrices had previously been determined for only 17 regulators for

10 which Applicants report motifs (Knuppel et al. J Comput Biol 1, 191-8 (1994)). In the case of Cin5, which showed the largest difference between the computationally derived motif (TTACRTAA) and the previously reported site (TTACTAA; Table 2), Applicants found that the motif that Applicants report is also the preferred in vitro target (Figure 7). Applicants supplemented the discovered motifs with additional

15 motifs from the literature that also passed conservation tests, and Applicants used this compendium of sequence motifs for 102 regulators (Table 3) in all subsequent analysis.

Example 2: Constructions of Transcriptional Regulatory Code

20   Applicants constructed an initial version of the transcriptional regulatory code by mapping on the yeast genome sequence the motifs that are bound by regulators at high confidence ($P \leq 0.001$) and that are conserved among sensu stricto Saccharomyces species (Figure 2; http://web.wi.mit.edu/fraenkel/regulatory_map). This map includes 3,353 interactions within 1,296 promoter regions. Maps of

25 regulatory sites encompassing larger numbers of promoters, constructed with lower confidence information, can also be viewed on the authors' website. Because the information used to construct the map includes binding data from multiple growth environments, the map describes transcriptional regulatory potential within the genome. During growth in any one environment, only subsets of the binding sites

30 identified in the map are occupied by transcriptional regulators, as Applicants describe in more detail below.

Where the functions of specific transcriptional regulators were established previously, the functions of the genes they bind in the regulatory map are highly consistent with this prior information. For example, the amino acid biosynthetic regulators Gcn4 and Leu3 bind to sites in the promoter of *BAP2* (chromosome II),

5   which encodes an amino acid transporter (Figure 2A). Six well-studied cell cycle transcriptional regulators bind to the promoter for *YHP1* (chromosome IV), which has been implicated in regulation of the G1 phase of the cell cycle. The regulator of respiration Hap5, binds upstream of *COX4* (chromosome VII), which encodes a component of the respiratory electron transport chain. Where regulators with

10   established functions bind to genes of unknown function, these target genes are newly implicated in such functional processes.

The utility of combining regulator binding data and sequence conservation data is illustrated in Figure 2B. All sequences matching the regulator DNA binding

15   specificities described in this study (Table 2) that occur within the 884 base-pair intergenic region upstream of the gene *BAP2* are shown in the upper panel. The subset of these sequences that have been conserved in multiple yeast species, and are thus likely candidates for regulator interactions, are shown in the middle panel. The presence of these conserved regulatory sites indicates the potential for regulation via

20   this sequence, but does not indicate whether the site is actually bound by a regulator under some growth condition. The incorporation of binding information (bottom panel) identifies those conserved sequences that are utilized by regulators in cells grown under the conditions examined.

25   The distribution of binding sites for transcriptional regulators reveals there are constraints on the organization of these sites in yeast promoters (Figure 2C). Binding sites are not uniformly distributed over the promoter regions, but rather show a sharply peaked distribution. Very few sites are located in the region 100 base pairs (bp) upstream of protein coding sequences. This region typically includes the

30   transcription start site and is bound by the transcription initiation apparatus. The vast majority (74%) of the transcriptional regulator binding sites lie between 100 and 500 bp upstream of the protein coding sequence, far more than would be expected at

random (53%). Regions further than 500 bp contain fewer binding sites than would be expected at random. It appears that yeast transcriptional regulators function at short distances along the linear DNA, a property that reduces the potential for inappropriate activation of nearby genes.

5

Applicants note that specific arrangements of DNA-binding site sequences occur within promoters, and suggest that these promoter architectures provide clues to regulatory mechanisms (Figure 3). For example, the presence of a DNA-binding site for a single regulator is the simplest promoter architecture and, as might be

10 expected, Applicants found that sets of genes with this feature are often involved in a common biological function (Table 4). A second type of promoter architecture consists of repeats of a particular binding site sequence. Repeated binding sites have been shown to be necessary for stable binding by the regulator Dal80 (Cunningham *et al. J Bacteriol* 175, 5851-61 (1993)). This repetitive promoter architecture can

15 also allow for a graded transcriptional response, as has been observed for the *HIS4* gene (Donahue *et al. Cell* 32, 89-98 (1983)). A number of regulators, including Dig1, Mbp1, and Swi6 show a statistically significant preference for repetitive motifs (Table 5). A third class of promoter contains binding sites for multiple different regulators. This promoter arrangement implies that the gene may be subject

20 to combinatorial regulation, and Applicants expect that in many cases the various regulators can be used to execute differential responses to varied growth conditions. Indeed, Applicants note that many of the genes in this category encode products that are required for multiple metabolic pathways and are regulated in an environment-specific fashion. In the fourth type of promoter architecture Applicants discuss here,

25 binding sites for specific pairs of regulators occur more frequently within the same promoter regions than would be expected by chance (Table 6). This "co-occurring" motif architecture implies that the two regulators physically interact or have shared functions at multiple genes.

30 Example 3: Identification of Conditions which alter the Distribution of Biologically-Active DNA-binding Sites

By conducting genome-wide binding experiments for some regulators under

multiple cell growth conditions, Applicants learned that regulator binding to a subset
of the regulatory sequences is highly dependent on the environmental conditions of
the cell (Figure 8). Applicants observed four common patterns of regulator binding
behavior (Figure 4, Table 7). Prior information about the regulatory mechanisms

5   employed by well-studied regulators in each of the four groups suggests hypotheses
to account for the environment-dependent binding behavior of the other regulators.

"Condition invariant" regulators bind essentially the same set of promoters
(within the limitations of noise) in two different growth environments (Figure 4).

10   Leu3, which is known to regulate genes involved in amino acid biosynthesis, is
among the best studied of the regulators in this group. Binding of Leu3 *in vivo* has
been shown to be necessary, but not sufficient for activation of Leu3-regulated genes
(Kirkpatrick *et al. Mol Cell Biol* 15, 4021-30 (1995)). Rather, regulatory control of
these genes requires association of a leucine metabolic precursor with Leu3 to

15   convert it from a negative to positive regulator. Applicants note that other zinc
cluster type regulators that show "condition invariant" behavior are known to be
regulated in a similar manner (Axelrod *et al. Mol Cell Biol* 11, 564-7 (1991), Ma *et
al. Cell* 50, 137-42 (1987)). Thus, it is reasonable to propose that the activation or
repression functions of some of the other regulators in this class will be independent

20   of DNA binding.

"Condition enabled" regulators do not bind the genome detectably under one
condition, but bind a substantial number of promoters with a change in environment.
Msn2 is among the best-studied regulators in this class, and the mechanisms

25   involved in Msn2-dependent transcription provide clues to how the other regulators
in that class may operate. Msn2 is known to be excluded from the nucleus when
cells grow in the absence of stresses, but accumulates rapidly in the nucleus when
cells are subjected to stress (Beck *et al. Nature* 402, 689-92 (1999), Chi *et al.
Genes Dev* 15, 1078-92. (2001)). This condition-enabled behavior was also observed

30   for the thiamine biosynthetic regulator Thi2, the nitrogen regulator Gat1, and the
developmental regulator Rim101. Applicants suggest that many of these
transcriptional regulators are regulated by nuclear exclusion or by another

mechanism that would cause this extreme version of condition-specific binding.

"Condition expanded" regulators bind to a core set of target promoters under
one condition, but bind an expanded set of promoters under another condition. Gcn4
5   is the best-studied of the regulators that fall into this "expanded" class. The levels of
Gcn4 are reported to increase 6-fold when yeast are introduced into media with
limiting nutrients (Albrecht et al. J Biol Chem 273, 12696-702. (1998)), due largely
to increased nuclear protein stability (Chi et al. Genes Dev 15, 1078-92. (2001),
Kornitzer et al. EMBO J 13, 6021-30. (1994)), and under this condition Applicants
10   find Gcn4 binds to an expanded set of genes. Interestingly, the probes bound when
Gcn4 levels are low contain better matches to the known Gcn4 binding site than
probes that are bound exclusively at higher protein concentrations, consistent with a
simple model for specificity based on intrinsic protein affinity and protein
concentration (Figure 9). The expansion of binding sites by many of the regulators
15   in this class may reflect increased levels of the regulator available for DNA binding.

"Condition altered" regulators exhibit altered preference for the set of
promoters bound in two different conditions. Ste12 is the best studied of the
regulators whose binding behavior falls into this "altered" class. Depending on the
20   interactions with other regulators, the specificity of Ste12 can change and alter its
cellular function (Zeitlinger, et al. Cell 113, 395-404 (2003). For example, under
filamentous growth conditions, Ste12 interacts with Tec1, which has its own DNA-
binding specificity (Baur et al. Mol Cell Biol 17, 4330-7 (1997)). This condition-
altered behavior was also observed for the transcriptional regulators Aft2, Skn7, and
25   Ume6. Applicants propose that the binding specificity of many of the transcriptional
regulators may be altered through interactions with other regulators or through
modifications (e.g., chemical) that are environment-dependent.

Substantial portions of eukaryotic genome sequence are believed to be
30   regulatory (Kellis et al. Nature 423, 241-54 (2003), Cliften et al. Science 301, 71-6
(2003), Waterston et al. Nature 420, 520-62 (2002)), but the DNA sequences that
actually contribute to regulation of genome expression have been ill-defined. By

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mapping the DNA sequences bound by specific regulators in various environments, Applicants identify the regulatory potential embedded in the genome and provide a framework for modeling the mechanisms that contribute to global gene expression. Applicants anticipate that the approaches used here to map regulatory sequences in

5   yeast can also be used to map the sequences that control genome expression in higher eukaryotes.

Table 1: List of regulators and environmental conditions examined*

A1	Dat1	Hap3	Met18	Pho4[11]	Sig1[1]	Swi4	YDR266C
Abf1	Dig1[5,6]	Hap4[2,3]	Met28[3]	Pip2	Sip3	Swi5	YDR520C
Abt1	Dot6	Hap5[3]	Met31[3]	Ppr1	Sip4[3]	Swi6	YER051W
Aca1	Ecm22	Hir1	Met32[3]	Put3[2,3]	Skn7[1,2,7]	Tbs1	YER130C
Ace2	Eds1	Hir2	Met4[3]	Rap1[3]	Sko1	Tec1[5,6]	YER184C
Adr1[3,7]	Fap7	Hir3	Mga1[1]	Rco1	Smk1	Thi2[12]	YFL044C
Aft2[1,2]	Fhl1[1,3,4]	Hms1	Mig1[8]	Rcs1[1,2,3]	Smp1	Tos8	YFL052W
Arg80[3]	Fkh1	Hms2	Mig2[1]	Rdr1	Snf1	Tye7	YGR067C
Arg81[3]	Fkh2[1,2]	Hog1	Mig3	Rds1[1]	Snt2	Uga3[3,4]	Yhp1
Aro80[3]	Fzf1	Hsf1[1,2,7]	Mot3[1,2,3]	Reb1[1,2]	Sok2[5]	Ume6[1]	YJL206C[1,2]
Arr1[1]	Gal3	Ifh1	Msn1	Rfx1	Spt10	Upc2	YKL222C
Ash1[5]	Gal4[8,9]	Ime1[1]	Msn2[1,2,4,7,10]	Rgm1	Spt2	Usv1	YKR064W
Ask10	Gal80	Ime4[1]	Msn4[1,2,4,10]	Rgt1[8]	Spt23	War1	YLR278C
Azf1	Gat1[3,4,7]	Ino2	Mss11[5]	Rim101[1,2]	Srd1	Wtm1	YML081W
Bas1[3]	Gat3	Ino4	Mth1[8]	Rlm1[5]	Stb1	Wtm2	YNR063W
Bye1	Gcn4[3,4]	Ixr1	Ndd1	Rlr1	Stb2	Xbp1[2,7]	Yox1
Cad1[1,3]	Gcr1	Kre33	Ndt80	Rme1	Stb4	Yap1[1,2,7]	YPR022C
Cbf1[3]	Gcr2[3]	Kss1[5,6]	Nnf2	Rox1[1,2]	Stb5	Yap3[1]	YPR196W
Cha4[3]	Gln3[3,4]	Leu3[3]	Nrg1[1,2]	Rph1[1,2,3]	Stb6	Yap5[1]	Yrr1
Cin5[1,2]	Gts1	Mac1[1]	Oaf1	Rpi1	Ste12[5,6]	Yap6[1,2]	Zap1
Crz1	Gzf3[1,4]	Mal13	Opi1	Rpn4[1,2]	Stp1[3]	Yap7[1,2]	Zms1
Cst6	Haa1	Mal33[1,2]	Pdc2	Rtg1[3,4]	Stp2	YBL054W	
Cup9	Hac1	Mbf1	Pdr1[2]	Rtg3[1,2,3,4]	Stp4	YBR239C	
Dal80[4]	Hal9	Mbp1[1,2]	Pdr3	Rts2	Sum1	YBR267W	
Dal81[3,4]	Hap1	Mcm1[5,6]	Phd1[5]	Sfl1	Sut1	YDR026C	
Dal82[3,4]	Hap2[4]	Mds3	Pho2[1,2,3,11]	Sfp1[1,2,3]	Sut2	YDR049W	

[1] Highly hyperoxic   [4] Nutrient deprived   [7] Heat   [10] Acidic

[2] Mildly hyperoxic   [5] Filamentation   [8] Galactose   [11] Phosphate deprived

[3] Amino acid starved   [6] Mating   [9] Raffinose   [12] Vitamin deprived

*All regulators were profiled in rich medium. A subset of these were profiled in at least one other environmental condition, as indicated. A complete description of the conditions can be found at the authors' website.

5

Table 2. Similarity of discovered specificities to literature

Regulator	Distance[1]	Discovered	Literature
Abf1	0.143	rTCAytnnnnAcg	rTCAyTnnnnACGw
Ace2	0.18	tGCTGGT	GCTGGT
Aft2	0.15	rCACCC	ATCTTCAAAAGTGCA- CCCATTTGCAGGTGC
Azf1	0.203	YwTTkcKkTyyckgykky	TTTTTCTT
Bas1	0.045	TGACTC	TGACTC
Cad1	0.089	mTTAsTmAkC	TTACTAA
Cbf1	0.105	tCACGTG	rTCACrTGA
Cin5	0.324	TTAcrTAA	TTACTAA
Fkh1	0.123	gtAAAcAA	GGTAAACAA
Fkh2	0.212	GTAAACA	GGTAAACAA
Gal4	0.11	CGGnnnnnnnnnncCg	CGGnnnnnnnnnnnCCG
Gat1	0.004	aGATAAG	GATAA
Gcn4	0.123	TGAsTCa	ArTGACTCw
Gln3	0.148	GATAAGa	GATAAGATAAG
Hap1	0.191	GGnnaTAnCGs	CGGnnnTAnCGG
Hap4	0.146	gnCcAAtcA	YCNNCCAATNANM
Hsf1	0.198	TTCynnnnnnTTC	TTCTAGAAnnTTCT
Ino2	0.236	CAcaTGc	ATTTCACATC
Ino4	0.163	CATGTGaa	CATGTGAAAT
Leu3	0.131	cCGgtacCGG	yGCCGGTACCGGyk
Mbp1	0.073	ACGCGt	ACGCGT
Mcm1	0.181	CCnrAtnngg	wTTCCyAAwnnGGTAA
Msn2	0.308	mAGGGGsgg	mAGGGG

Nrg1	0.042	GGaCCCT	CCCT
Pdr1	0.301	ccGCCgRAwr	CCGCGG
Pho4	0.096	CACGTGs	cacgtkng
Rap1	0.181	cayCCrtrCa	wrmACCCATACAyy
Rcs1	0.184	ggGTGcant	AAmTGGGTGCAkT
Reb1	0.055	TTACCCG	TTACCCGG
Rpn4	0.049	GGTGGCAAA	GGTGGCAAA
Sip4	0.184	CGGnynAATGGrr	yCGGAyrrAwGG
Skn7	0.228	GnCnnGsCs	ATTTGGCyGGsCC
Stb5	0.058	CGGnstTAta	CGG
Ste12	0.087	tgAAAC	ATGAAAC
Sum1	0.221	gyGwCAswaaw	AGyGwCACAAAAk
Sut1	0.295	gcsGsgnnsG	CGCG
Swi4	0.122	CgCsAAA	CnCGAAA
Swi6	0.214	CGCgaaa	CnCGAAA
Tec1	0.064	CATTCyy	CATTCy
Tye7	0.193	tCACGTGa	CAnnTG
Ume6	0.16	taGCCGCCsa	wGCCGCCGw
Yap1	0.124	TTaGTmAGc	TTAsTmA
Yap7	0.15	mTkAsTmA	TTACTAA
Zap1	0.085	ACCCTmAAGGTyrT	ACCCTAAAGGT

[1]Distance from known specificity was computed using the distance metric described in the experimental procedures.

5   Table 3 Regulator specificities

Regulator	Discovered specificity[1]	Known specificity[1,2]	Programs[3]
Abf1	rTCAytnnnnAcg	rTCAyTnnnnACGw	A, C, D, K, M, N
Ace2	tGCTGGT	GCTGGT	K
Adr1		GGrGk	
Aft2	rCACCC	...AAAGTGCACCCATT...	A, C, D, M, N

Regulator	Discovered specificity[1]	Known specificity[1,2]	Programs[3]
Arr1		TTACTAA	
Ash1		yTGACT	
Azf1	YwTTkcKkTyyckgykky	TTTTTCTT	N
Bas1	TGACTC	TGACTC	A, K, M, N
Cad1	mTTAsTmAkC	TTACTAA	A, C, D, M, N
Cbf1	tCACGTG	rTCACrTGA	A, C, D, K, M, N
Cin5	TTAcrTAA	TTACTAA	A, C, D
Dal80		GATAA	
Dal81		AAAAGCCGCGGGCGGGATT	
Dal82	GATAAG		D, K
Dig1	TgAAAca		A, C, D, K, M, N
Fhl1	TGTayGGrtg		A, C, D, K, M, N
Fkh1	gtAAAcAA	GGTAAACAA	A, C, D, K, M, N
Fkh2	GTAAACA	GGTAAACAA	A, C, D, K, M, N
Gal4	CGGnnnnnnnnnnncCg	CGGnnnnnnnnnnnCCG	A, K
Gal80		CGGnnnnnnnnnnnCCG	
Gat1	aGATAAG	GATAA	K
Gcn4	TGAsTCa	ArTGACTCw	A, C, D, K, M, N
Gcr1		GGCTTCCwC	
Gln3	GATAAGa	GATAAGATAAG	C, D, K
Gzf3		GATAAG	
Hac1		kGmCAGCGTGTC	
Hap1	GGnnaTAnCGs	CGGnnnTAnCGG	C, M
Hap2		CCAAT	
Hap3		CCAAT	
Hap4	gnCcAAtcA	YCNNCCAATNANM	A, C, D, M, N
Hap5		CCAAT	
Hsf1	TTCynnnnnnTTC	TTCTAGAAnnTTCT	A, C, D, K, M, N
Ime1	AAkGAAAnkwA		A
Ino2	CAcaTGc	ATTTCACATC	C, D, M, N
Ino4	CATGTGaa	CATGTGAAAT	A, C, D, K, M, N

Regulator	Discovered specificity[1]	Known specificity[1,2]	Programs[3]
Leu3	cCGgtacCGG	yGCCGGTACCGGyk	A, D, K, M
Mac1		GAGCAAA	
Mbp1	ACGCGt	ACGCGT	A, C, D, K, M, N
Mcm1	CCnrAtnngg	wTTCCyAAwnnGGTAA	A, C, D, M, N
Met31		AAACTGTGG	
Met32		AAACTGTGG	
Met4	RMmAwsTGKSgyGsc		C
Mot3		yAGGyA	
Msn2	mAGGGGsgg	mAGGGG	M
Msn4		mAGGGG	
Ndd1	CCnrAwnnGG		A, D
Nrg1	GGaCCCT	CCCT	A, C, D, M, N
Opi1		TCGAAyC	
Pdr1	ccGCCgRAwr	CCGCGG	M
Pdr3		TCCGCGGA	
Phd1	scnGCngg		A, D, N
Pho2	SGTGCGsygyG		N
Pho4	CACGTGs	cacgtkng	D, K, N
Put3		CGGnnnnnnnnnnCCG	
Rap1	cayCCrtrCa	wrmACCCATACAyy	A, C, D, M, N
Rcs1	ggGTGcant	AAmTGGGTGCAkT	C, D, M, N
Rds1	kCGGCCGa		D, N
Reb1	TTACCCG	TTACCCGG	A, C, D, K, M, N
Rfx1	TTgccATggCAAC		D
Rgt1		CGGAnnA	
Rim101		TGCCAAG	
Rlm1		CTAwwwwTAG	
Rlr1	ATTTTCnnCwTt		N
Rox1		ysyATTGTT	
Rph1		CCCCTTAAGG	
Rpn4	GGTGGCAAA	GGTGGCAAA	A, C, D, K, M, N

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Regulator	Discovered specificity[1]	Known specificity[1,2]	Programs[3]
Rtg3		GGTCAC	
Sfp1	ayCcrtACay		A, C, D, M, N
Sig1	ArGmAwCrAmAA		M
Sip4	CGGnynAATGGrr	yCGGAyrrAwGG	D
Skn7	GnCnnGsCs	ATTTGGCyGGsCC	A, C, D, M, N
Sko1		ACGTCA	. .
Smp1		ACTACTAwwwwTAG	
Snt2	yGGCGCTAyca		A, C, D, M, N
Sok2	tGCAgnna		A
Spt2	ymtGTmTytAw		M
Spt23	rAAATsaA		C
Stb1	rracGCsAa		C, D, K, M, N
Stb4	TCGgnnCGA		K
Stb5	CGGnstTAta	CGG	D, N
Ste12	tgAAAC	ATGAAAC	A, C, D, K, M, N
Stp1		rCGGCnnnrCGGC	
Sum1	gyGwCAswaaw	AGyGwCACAAAAk	A, C, D, M, N
Sut1	gcsGsgnnsG	CGCG	A, D, M
Swi4	CgCsAAA	CnCGAAA	A, C, D, K, M, N
Swi5		kGCTGr	
Swi6	CGCgaaa	CnCGAAA	A, C, D, M, N
Tec1	CATTCyy	CATTCy	C
Thi2	gmAAcyntwAgA		C, D
Tye7	tCACGTGa	CAnnTG	A, C, D, M
Uga3		CCGnnnnCGG	
Ume6	taGCCGCCsa	wGCCGCCGw	A, C, D, K, M, N
Xbp1		CTTCGAG	
Yap1	TTaGTmAGc	TTAsTmA	A, C, D, M
Yap3		TTACTAA	
Yap5		TTACTAA	
Yap6		TTACTAA	

Regulator	Discovered specificity[1]	Known specificity[1,2]	Programs[3]
Yap7	mTkAsTmA	TTACTAA	A, C, D, M, N
YDR026C	tTACCCGGm		C, D, M, N
Yhp1		TAATTG	
Yox1		YAATA	
Zap1	ACCCTmAAGGTyrT	ACCCTAAAGGT	N

[1]Text representation of the probability matrices. Lowercase letters indicate a weaker
preference (less information content at that position of the probability matrix).
Ambiguity Codes: S = C or G, W = A or T, R = A or G, Y = C or T, K = G or T,
M = A or C, n = A, C, G or T.

[2] Known specificities are taken from the YPD, SCPD, and TRANSFAC databases.

[3] Program Codes: A = AlignACE, C = CONVERGE, D = MDscan, K = Kellis *et al.*,
M = MEME, N = MEME_c.

Table 4 Overrepresented MIPS categories among single-regulator architecture
binding targets

Regulator	P value[1]	Enriched MIPS category[2]
Bas1	6.10e-09	nucleotide metabolism*
Fhl1	1.73e-15	ribosome biogenesis
Gal4	2.18e-04	C-compound and carbohydrate metabolism*
Gat1	4.92e-05	nitrogen and sulfur metabolism*
Gat1	2.63e-02	mRNA transcription*
Gat1	4.38e-02	amino acid metabolism
Gcn4	8.72e-12	amino acid metabolism*
Gzf3	2.21e-02	transport mechanism
Hap3	6.03e-03	lipid, fatty-acid and isoprenoid metabolism
Hap3	1.61e-02	allantoin and allantoate transporters
Hap3	2.50e-02	other energy generation activities
Hap4	3.33e-10	respiration

Hap4	1.78e-05	mitochondrial transport
Hap4	1.03e-02	transport mechanism
Hap4	2.12e-02	assembly of protein complexes
Hsf1	6.58e-06	stress response*
Ino4	5.31e-03	lipid, fatty-acid and isoprenoid metabolism*
Mbp1	1.04e-04	DNA processing
Met32	1.13e-04	amino acid metabolism*
Met32	1.21e-03	nitrogen and sulfur metabolism*
Met32	4.64e-02	amino-acid transporters
Mot3	3.89e-02	DNA processing
Msn2	4.40e-02	metabolism of energy reserves (glycogen, trehalose)
Put3	3.45e-02	other transport facilitators
Reb1	2.09e-05	vesicular transport (Golgi network, etc.)
Rfx1	3.57e-02	other protein-synthesis activities
Rox1	3.43e-02	cell death
Rpn4	2.49e-13	proteolytic degradation*
Rtg3	8.50e-03	other transcription activities
Sig1	2.97e-02	cell cycle
Sip4	2.69e-03	glyoxylate cycle
Sip4	1.57e-02	glycolysis and gluconeogenesis
Stb4	4.02e-02	allantoin and allantoate transporters
Stb5	2.42e-02	electron transport and membrane-associated energy conservation
Ste12	5.56e-03	cell differentiation*
Sut1	5.37e-03	glyoxylate cycle
Swi6	7.96e-03	nitrogen and sulfur metabolism
Thi2	1.15e-02	mRNA transcription*
Thi2	2.45e-02	metabolism of vitamins, cofactors, and prosthetic groups

[1]P values represent the probability, based on the hypergeometric distribution, of finding the observed number of genes (or more) with the specified MIPS Level 2 category under the null hypothesis that the genes were selected at random. The

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values have been corrected for testing multiple categories using Bonferroni correction.

[2]An asterisk (*) indicates that the category is also associated with the regulator itself.

Table 5 Regulators with a preference for repetitive motifs

Regulator	P value[1]	Non-repetitive	Repetitive
Dig1	1.43e-08	O: 25 E: 45	O: 38 E: 17
Mbp1	2.99e-08	O: 34 E: 56	O: 44 E: 21
Swi6	7.36e-06	O: 34 E: 50	O: 37 E: 20
Sok2	1.34e-05	O: 13 E: 24	O: 21 E: 9
Bas1	2.84e-04	O: 6 E: 12	O: 12 E: 5
Ste12	5.57e-04	O: 48 E: 62	O: 39 E: 24
Swi4	7.29e-04	O: 27 E: 38	O: 26 E: 14
Phd1	7.89e-03	O: 15 E: 21	O: 15 E: 8
Aft2	9.73e-03	O: 22 E: 29	O: 19 E: 11
Swi5	1.05e-02	O: 11 E: 16	O: 12 E: 6
Sfp1	3.03e-02	O: 7 E: 10	O: 8 E: 4
Ino2	4.77e-02	O: 11 E: 15	O: 10 E: 5

[1]P values represent the one-tailed probability, based on the chi-square distribution, of finding the observed number of non-repetitive and repetitive motif architecture promoters under the null hypothesis that the distribution for each regulator is the same as the average distribution for all regulators. O = observed number of occurrences; E = expected number of occurrences.

Table 6 Co-occurring regulator pairs[1]

Ace2, Fkh2	Dig1, Swi4	Mbp1, Stb1	Rlm1, Sko1
Ace2, Swi5	Dig1, Swi6	Mbp1, Swi4	Rox1, Sut1
Aft2, Rcs1	Dig1, Tec1	Mbp1, Swi6	Sip4, Stp1
Arr1, Yap3	Fhl1, Rap1	Mcm1, Ndd1	Skn7, Sok2

Azfl, Gzf3	Fhll, Sfpl	Mcml, Ste12	Skn7, Sutl
Basl, Met4	Fkhl, Fkh2	Mcml, Swi4	Skn7, Swi6
Cadl, Yapl	Fkh2, Mcml	Mcml, Swi6	Skn7, Xbpl
Cadl, Yap7	Fkh2, Nddl	Mcml, Tecl	Skol, Sok2
Cbfl, Met31	Fkh2, Swi6	Met31, Met32	Sok2, Sutl
Cbfl, Met32	Gatl, Spt23	Met31, Met4	Sok2, Swi6
Cbfl, Met4	Gcn4, Gln3	Met32, Met4	Spt23, Yoxl
Cbfl, Pho4	Gcn4, Leu3	Mot3, Roxl	Stbl, Swi4
Cbfl, Tye7	Gcrl, Tye7	Mot3, Skn7	Stbl, Swi6
Cin5, Phdl	Gln3, Hap2	Msn2, Msn4	Stbl, Tecl
Cin5, Skn7	Gzf3, Pdrl	Msn4, Nrgl	Ste12, Swi4
Cin5, Sok2	Hap2, Hap3	Nrgl, Rlml	Ste12, Swi6
Cin5, Sutl	Hap2, Hap4	Nrgl, Skn7	Ste12, Tecl
Cin5, Xbpl	Hap2, Hap5	Phdl, Roxl	Swi4, Swi6
Cin5, Yap6	Hap3, Hap5	Phdl, Skn7	Swi4, Tecl
Dal82, Gatl	Hap4, Hap5	Phdl, Sok2	Swi6, Tecl
Dal82, Gln3	Hsfl, Msn4	Phdl, Sutl	Yapl, Yap7
Dal82, Hap2	Ino2, Ino4	Phdl, Swi6	Yap6, Yap7
Digl, Mcml	Ino4, Skol	Rapl, Sfpl	
Digl, Ste12	Macl, Rcsl	Rim101, Yoxl	

[1]Shown are co-occurring regulator pairs ($P \leq 0.005$). P values represent the probability, based on the hypergeometric distribution, of finding the observed number of intergenic regions (or more) bound by both regulators under the null hypothesis that binding for the two regulators is independent.

Table 7 Behavior classifications of regulators[1]

Invariant[2]	Enabled[3]	Expanded[4]	Altered[5]
Fhll	Adrl	Basl	Adrl
Gal4	Arrl	Cadl	Aft2
Gcn4	Ashl	Cbfl	Cadl
Hsfl	Dal81	Cin5	Cin5
Leu3	Fhll	Dal82	Dal80
Put3	Gatl	Fkh2	Dal82
Ste12	Hap4	Gal4	Digl

Ume1	Hsf1	Gcn4	Fkh2
Yap7	Mot3	Gln3	Gat1
	Msn2	Hap2	Gln3
	Pdr1	Mac1	Gzf3
	Phd1	Mbp1	Hap4
	Pho2	Mcm1	Hap5
	Put3	Met31	Mbp1
	Rap1	Met32	.Mot3
	Rgt1	Met4	Msn2
	Rim101	Nrg1	Msn4
	Rlm1	Rcs1	Phd1
	Rph1	Rds1	Pho4
	Rpn4	Reb1	Reb1
	Rtg3	Rox1	Rox1
	Sfp1	Rpn4	Rtg3
	Sig1	Rtg3	Skn7
	Sip4	Skn7	Ste12
	Sok2	Ste12	Tec1
	Stp1		Ume6
	Thi2		Yap1
	Uga3 ·		Yap6
	Xbp1		
	Yap1		
	Yap7		

[1] The binding of each regulator was compared in pairwise fashion for every environmental condition in which that regulator was profiled. Some regulators fall into multiple categories depending on exactly which conditions are compared.

[2] The ratio of the overlap of bound probes for a regulator ($P \leq 0.001$) was greater than 0.66 and the ratio of the number of bound probes was between 0.66 and 1.5.

[3] Regulator bound to no probes in one environment.

[4] The ratio of the overlap of bound probes for a regulator was greater than 0.66 and the ratio of the number of bound probes was less than 0.66 or greater than 1.5.

[5] Regulator bound at least one probe in both environments and the ratio of the overlap of bound probes was less than 0.66.

Table 8. Motif score significance cutoffs ($P \leq 0.001$)

Enrichment Score[1]

Number of sequences	Converge	AlignACE	MDscan	MEME	MEME_c

10	12.70	20.32	11.78	13.54	n/a
20	11.96	21.14	12.95	12.89	9.81
30	11.43	20.43	13.30	12.57	n/a
40	11.34	20.62	14.04	11.64	7.53
50	10.74	19.94	12.23	12.81	7.43
60	10.50	19.71	10.95	12.37	n/a
70	10.34	18.30	13.25	11.34	n/a
80	10.20	19.40	12.84	11.93	n/a
100	9.36	20.31	11.56	10.58	2.91
120	n/a	18.59	13.14	10.94	n/a
140	8.14	18.52	11.26	10.87	n/a
160	n/a	20.04	11.38	9.77	n/a

ROC a.u.c.[1]

Number of sequences	Converge	AlignACE	MDscan	MEME	MEME_c
10	n/a	n/a	n/a	n/a	n/a
20	0.812	0.842	0.857	0.925	n/a
30	0.758	0.773	0.793	0.831	0.785
40	0.720	0.713	0.758	0.764	0.737
50	0.687	0.674	0.719	0.737	0.711
60	0.670	0.662	0.688	0.706	0.654
70	0.663	0.641	0.686	0.684	0.664
80	0.643	0.626	0.670	0.675	0.648
100	0.634	0.615	0.664	0.633	0.606
120	0.624	0.604	0.629	0.624	0.602
140	0.608	n/a	0.634	n/a	0.590
160	0.594	0.580	0.613	0.593	0.588

[1]Motif score significance $P \leq 0.001$ thresholds for "Enrichment" and "ROC a.u.c."

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specificity metrics obtained from calculations on randomized selections of intergenic regions as described in Methods. Entries containing "n/a" denote that the empirical distribution was not normal. The threshold for the CC4 metric (4.95) is not dependent on the number of sequences.